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March 11, 1999

Box Patent Application
Assistant Commissioner for Patents
Washington, D.C. 20231

Re: U.S. Continuation-in-Part Patent Application
for **TERTIARY STRUCTURE OF PEANUT ALLERGEN ARA H 1**
Atty. Dkt. No.: ARK00898103A

Dear Sir:

Enclosed, please find a patent application in the above, along with the following:

1. Declaration and Power of Attorney;
2. Verified Statement Claiming Small Entity Status for Inventor;
3. Verified Statement Claiming Small Entity Status for Nonprofit Organization;
4. One copy of drawings (64 Sheets); and,
6. Check in the amount of $380.00 to cover the filing fee.

This patent application consists of 128 pages of specification, Sequence Listing, Abstract, and contains 26 claims, including 15 independent claims.

Please charge any deficiency in fees or credit any overpayment in fees associated with this application to Deposit Account No. 08-1500.

Respectfully submitted,

HEAD, JOHNSON & KACHIGIAN

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Date: March 11, 1999

Applicant: Burks et al. Atty. Dkt. No.
Serial No: ARK00898103A
Filing Date:
Title: **TERTIARY STRUCTURE OF PEANUT ALLERGEN ARA H 1**

VERIFIED STATEMENT (DECLARATION)
CLAIMING SMALL ENTITY STATUS
[37 CFR SECTION 1.9 (f) and SECTION 1.27 (b)]
INDEPENDENT INVENTOR(S)

As a below named inventor, I hereby declare that I qualify as an independent inventor as defined in 37 CFR 1.9 (c) for purposes of paying reduced fees under Section 41 (a) and (b) of Title 35, United States Code, to the Patent and Trademark Office with regard to the invention entitled:

TERTIARY STRUCTURE OF PEANUT ALLERGEN ARA H 1

described in:

[X] the specification filed herewith.
[ ] Application Serial No. _____ filed _____.
[ ] Patent No. _____ issued _____.

I have not assigned, granted, conveyed or licensed and am under no obligation under contract or law to assign, grant, convey or license any rights in the invention to any person who could not be classified as an independent inventor under 37 CFR 1.9 (c) if that person had made the invention, or to any concern which would not qualify as a small business concern under 37 CFR 1.9 (d) or a nonprofit organization under 37 CFR 1.9 (e).

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[x] persons, concerns or organizations listed below*

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FULL NAME: University of Arkansas [ ] INDIVIDUAL
ADDRESS: 2404 N. University Ave. [ ] SMALL BUSINESS CONCERN
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I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. [37 CFR 1.28(b)].

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

NAMES OF INVENTORS

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Gael Cockrell	Date
Gary A. Bannon, Ph.D.	Date
J. Steven Stanley, Ph.D.	Date
David S. Shin	Date
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Shau K. Huang	Date
Soheila J. Maleki	Date
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Atty. Dkt. No.
ARK00898103A

Applicant : Burks et al.
Serial No. :
Filing Date:
Title : **TERTIARY STRUCTURE OF PEANUT ALLERGEN ARA H 1**

USA PATENT AND TRADEMARK OFFICE
VERIFIED DECLARATION CLAIMING SMALL ENTITY STATUS
37 CFR 1.9(f) and 1.27(d) - NONPROFIT ORGANIZATION

I hereby declare that I am an official empowered to act on behalf of the nonprofit organization identified below:

NAME OF ORGANIZATION University of Arkansas
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TYPE OF ORGANIZATION:

[X] University or other institution of higher education

[ ] Tax exempt under Internal Revenue Service Code (26 USC 501(a) and 501(c)(3))

[ ] Nonprofit scientific or educational under statute of state of the United States of America
State: _____
Citation of statute: _____

[ ] Would qualify as tax exempt under Internal Revenue Service Code (26 UCS 501(a) and 501(c)(3)) if located in the United States of America

[ ] Would qualify as nonprofit scientific or educational under statute of state of the United States of America if located in the United States of America
State: _____
Citation of statute: _____

I hereby declare that the nonprofit organization identified above qualifies as a nonprofit organization as defined in 37 CFR 1.9(e) for purposes of paying reduced fees under Section 41(a) and (b) of Title 35, United States Code with regard to the invention entitled **TERTIARY STRUCTURE OF PEANUT ALLERGEN ARA H 1** by inventor(s) A. Wesley Burks, Jr., Ricki M. Helm, Ph.D., Gael Cockrell, Gary A. Bannon, Ph.D., J. Steven Stanley, David S. Shin, Hugh Sampson, Cesar M. Compadre, Shau K. Huang, Soheila J. Maleki, and Randall A. Kopper described in

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[X] the specification filed herewith.
[ ] application Serial No. _____, filed _____.
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I hereby declare that rights under contract or law have been conveyed to and remain with the nonprofit organization with regard to the above-identified invention.

If the rights held by the nonprofit organization are not exclusive, each individual, concern or organization having rights to the invention is listed below* and no rights to the invention are held by any person, other than the inventor, who could not qualify as a small business concern under 37 CFR 1.9(d) or by any concern which would not qualify as a small business concern under 37 CFR 1.9(d) or a nonprofit organization under 37 CFR 1.9(e).

*NOTE: Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities. (37 CFR 1.27).

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SIGNATURE: _____ DATE: March 10, 1999

UNITED STATES PATENT APPLICATION

FOR

TERTIARY STRUCTURE OF PEANUT ALLERGEN ARA H 1

TERTIARY STRUCTURE OF PEANUT ALLERGEN ARA H 1

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation-in-part of U.S. provisional application Serial No. 60/077,763, filed on March 12, 1998.

Allergy to peanut is a significant health problem because of the prevalence and potential severity of the reaction. Ara h 1, a major peanut allergen, has been isolated and characterized and was shown to consist of 626 amino acids and contain 23 linear IgE-binding epitopes, 6-10 residues in length. The amino acids important for peanut-specific IgE binding were determined by synthesizing wild type and mutant peptides with single alanine, glycine or methionine substitutions at each position followed by incubation in pooled serum from patients with peanut hypersensitivity. From this analysis it was determined that amino acids which reside in the midele of the epitope were generally more critical for IgE binding. Furthermore, though polar charged residues occur most frequently within the epitopes, apolar residues were found to be more important for IgE binding. In addition, it was found that each epitope could be mutated resulting in loss of ability to bind IgE with only a single amino acid substitution. To further characterize the epitopes a homology-based molecular model of the Ara h 1 protein was made. The model represents residues 171-586 allowing visualization of epitopes 10-22. The majority of these epitopes appear to be clustered to certain areas of the molecule. Many of the critical amino acids involved in binding are evenly distributed on the surface and not buried in the hydrophobic core. The information from the mutational analysis along with the molecular model aids in the design of immunotherapies.

Biochemical And Structural Analysis Of The IgE Binding Sites On Ara h 1, An Abundant and Highly Allergenic Peanut Protein

Allergy to peanut is a significant IgE[1]-mediated health problem because of the high prevalence, potential severity, and chronicity of the reaction. Ara h 1, an abundant peanut protein, is recognized by serum IgE from >90% of peanut sensitive individuals. It has been shown to belong to the vicilin family of seed storage proteins and contain 23 linear IgE-binding epitopes. In this communication we have determined the critical amino acids within each of the IgE binding epitopes of Ara h 1 that are important for immunoglobulin binding. Surprisingly, substitution of a single amino acid within each of the epitopes led to loss of IgE binding. In addition, hydrophobic residues appeared to be most critical for IgE binding. The position of each of the IgE binding epitopes on a homology-based molecular model of Ara h 1 showed that they were clustered into two main regions, despite their more even distribution in the primary sequence. Finally, we have shown that Ara h 1 forms a stable trimer by the use of a reproducible fluorescence assay. This information is important in studies designed to reduce the risk of peanut-induced anaphylaxis by lowering the IgE binding capacity of the allergen.

INTRODUCTION

It is estimated that up to 8% of children and 2% of adults have allergic reactions to foods (1-3). Peanut allergy is one of the most common and serious of the immediate hypersensitivity reactions to foods in terms of persistence and severity of reaction. Unlike the clinical symptoms of other food allergies, the reactions to peanuts are rarely outgrown, therefore, most diagnosed children will have the disease for a lifetime (4,5). In a sensitized individual, ingestion of peanuts results in mast cell bound IgE binding to a specific allergen.

The IgE-allergen complex causes mast cell receptors to cross-link, inducing a signal transduction cascade that ends in degranulation, and release of a variety of mediators that give rise to the clinical symptoms of peanut hypersensitivity (6,7). The majority of cases of fatal food-induced anaphylaxis involve ingestion of peanuts (8,9). Currently, the only effective treatment for food allergy is avoidance of the food. For peanut allergic individuals total avoidance is difficult as peanuts are increasingly being used in the diet as an economical protein source in processed foods.

Because of the significance of the allergic reaction and the widening use of peanuts as protein extenders in processed foods, there is increasing interest in defining the allergenic proteins and exploring ways to decrease the risk to the peanut-sensitive individual. Various studies over the last several years have identified the major allergens in peanuts as belonging to different families of seed storage proteins (10,11). For example, two of the major peanut allergens Ara h 1 and Ara h 2 belong to the vicilin and conglutin families of seed storage proteins, respectively. The vicilins represent one of the most abundant proteins found in legumes used for human consumption. This class of proteins does not have any known enzymatic activity but are thought to interact with each other to form unique higher order oligomeric structures which may help in packaging these proteins into seeds (12). Because the vicilins represent such a large percentage of the total protein in a seed, any approach designed to alter the IgE binding capacity of this protein would require that the genetically engineered gene product retain its native function, properties and three-dimensional structure.

Genetically modified plants are being used more frequently as food sources for

human consumption. The major emphasis has been on the introduction of genes whose products would enhance the nutritional value or disease resistance of the transgenic plant. One of the major concerns of this approach is that a gene will be introduced that encodes an unwanted or unknown allergen that would put allergic individuals at risk. Indeed, the introduction of a gene encoding a major Brazil nut allergen into soybeans, ostensibly to increase the nutritional value of soybeans, is a prime example (13). In cases where allergens are transferred into plants, consumers must be informed of the existence of the allergen by labeling as suggested by the United States Food and Drug Administration. In addition, a range of tests that compare the physicochemical properties of known allergens with expressed trangenic products has been proposed for those gene products of unknown allergenicity (14-16). Currently, there is little known about the physicochemical properties of many of the plant allergens and there have been few investigations aimed at modifying allergenic proteins.

Previous work on the allergenic aspects of the Ara h 1 protein has shown that it is recognized by serum IgE from >90% of peanut sensitive individuals, indicating that it is a major allergen involved in the clinical etiology of this disease (17). Recently, using pooled serum IgE from a population of peanut hypersensitive individuals, 23 linear IgE binding epitopes of this allergen have been mapped (10). There was no obvious sequence motif shared by the epitopes. In this communication we have determined the critical amino acids within each of the IgE binding epitopes of Ara h 1 that are important to immunoglobulin binding. Surprisingly, substitution of a single amino acid within each of the epitopes led to loss of IgE binding. In addition, the hydrophobic residues located in the center of the

epitope appeared to be most critical to IgE binding. The position of each of the IgE binding epitopes on a homology-based tertiary structure model of Ara h 1 showed that they were clustered into two main regions. This was in contrast to previous observations that showed the IgE binding epitopes distributed along the linear sequence of the molecule. Finally, we have shown that, like other vicilins, Ara h 1 forms a stable trimer by the use of a reproducible fluorescence assay. This assay will allow for the rapid assessment of the effect that amino acid changes in Ara h 1 primary sequence have on tertiary structure. This information will be important in studies designed to reduce the risk of peanut-induced anaphylaxis by lowering the IgE binding capacity of the allergen.

METHODS

Serum IgE. Serum from 15 patients with documented peanut hypersensitivity reactions (mean age, 25 yrs) was used to determine relative binding affinities between wild type and mutant Ara h 1 synthesized epitopes. The patients had either a positive double-blind, placebo-controlled, food challenge (DBPCFC) or a convincing history of peanut anaphylaxis (laryngeal edema, severe wheezing, and/or hypotension; 18). At least 5 ml of venous blood was drawn from each patient, allowed to clot, and serum was collected. A serum pool from 12 to 15 patients was made by mixing equal aliquots of serum IgE for our experiments. The pools were then used in immunoblot analysis. All studies were approved by the Human Use Advisory Committee at the University of Arkansas for Medical Sciences.

Peptide synthesis. Individual peptides were synthesized on a derivatized cellulose membrane using 9-fluorenylmethoxycarbonyl (Fmoc) amino acid active esters according to the manufacturer=s instructions (Genosys Biotechnologies, Woodlands, Texas; 19). Peptide

synthesis reactions were monitored by bromophenol blue color reactions during certain steps of synthesis. Cellulose derivitised membranes and Fmoc-amino acids were supplied by Genosys Biotechnologies. All other chemicals were purchased from Aldrich Chemical Company, Inc. (Milwaukee, WI) or Fluka (Buchs, Switzerland). Membranes were either probed immediately or stored at -20°C until needed.

IgE binding assays. Cellulose membranes containing synthesized peptides were washed 3 times in Tris-buffered saline (TBS; 136 mM NaCl, 2.7 mM KCl, and 50 mM trizma base pH 8.0) for 10 min at room temperature and then incubated overnight in blocking buffer: [TBS, 0.05% tween 20; concentrated membrane blocking buffer supplied by Genosys; and sucrose (9.0:1.0:0.5)]. The membrane was then incubated in pooled sera diluted 1:5 in 20mM Tris-Cl pH7.5, 150mM NaCl, and 1% bovine serum albumin overnight at 4° C. Primary antibody was detected with ^{125}I-labeled equine anti-human IgE (Kallestad. Chaska, MN), followed by autoradiography.

Quantitation of IgE binding. Relative amounts of IgE binding to individual peptides were determined by scanning autoradiographs using a Bio-Rad (Hercules, CA) model GS-700 imaging laser densitometer and quantitated with Bio-Rad molecular analyst software. A background area was scanned and subtracted from the obtained values. Following quantitation, wild type intensities were normalized to a value of one and the mutants were calculated as percentages relative to the wild type.

***Homology based model of* Ara h 1.** Molecular modeling and computations were performed on Silicon Graphics workstations running IRIX 6.2. The Wisconsin Genetic Computer Group (GCG) software package (20) was also used on a digital ALPHA

workstation using OpenVMS v6.1. The X-ray crystal structure of the phaseolin A chain[2] (PDB #2PHL A, 2.2 D resolution) from *Phaseolus vulgaris* was used as the template for homology-based modeling (12,21,22). Ara h 1 was modeled as a monomer using the COMPOSER module of SYBYL version 6.3 from Tripos Inc. (St. Louis, MO). Phaseolin is a smaller protein than Ara h 1 and it only allowed for the modeling of the region between residues 172-586. Residues Ser211 - Asp219 and Asn281 - Lys282 on the structure of phaseolin were not solved because of low electron density (12). Before attempting to use the structure for modeling, the regions were constructed using the protein loop search option in SYBYL and minimized using local annealing and the Powell algorithm.

Alignment between Ara h 1 and phaseolin was determined using COMPOSER and was optimized with information from alignment of Ara h 1 to other vicilin homologs using the GCG pileup program. Following alignment, structurally conserved regions were constructed. Loops were then added using orientations to fragments from x-ray crystal structures in the SYBYL database following homology searches and fitting screens. The model was minimized with the CHARMM force field using the Adopted Basis Newton-Raphson method using QUANTA version 96 from Molecular Simulations Inc./BIOSYM (Burlington, MA). The protein backbone was given a harmonic force constraint constant of 500 to hold it rigid during the first 400 iterations of minimization, followed by relaxation with 100 steps each at constraints of 400, 300, 200, 100 and a final 400 steps with a constraint of 10 (23,24).

Fluoresence anisotropy of* Ara h 1 *higher order structure. Ara h 1 was purified to >95% homogeneity from crude peanut extract and labeled with flourescein[3]. A constant amount

of the labeled protein, 10 nM, in binding buffer (50 mM Tris, 1mM EDTA, 100mM NaCl, 2mM DTT, 5% glycerol, pH 7.5) was mixed with serial dilutions (by 0.5 or 0.8 increments) of unlabeled Ara h 1 to analyze oligomer formation. Fluorescence measurements were made using a Beacon fluorescence polarization spectrometer (Pan Vera, Madison, WI) with fixed excitation (490 nm) and emission (530 nm) wavelengths at room temperature (24 °C) in a final volume of 1.1 ml (25,26). Each data point is an average of three independent measurements. The intensity of fluorescence remained constant throughout the anisotropy measurements.

Cross linking experiments: Cross linking experiments were done exactly as described in Maleki et al. (27). Briefly, proteins were desalted into PBS, pH 8.0 using disposable PD-10 gel filtration columns. The protein cross-linking reagent utilized was dithiobis(succinimidyl propionate) (DSP). Limited cross-linking was performed so the monomer disappearance could be observed and to minimize the formation of nonspecific complexes.

RESULTS

IgE-binding characteristics of the **Ara h 1** *epitopes.* The amino acids essential to IgE binding in the Ara h 1 epitopes were determined by synthesizing duplicate peptides with single amino acid changes at each position. These peptides were then probed with pooled serum IgE from 15 patients with peanut hypersensitivity to determine if the changes affected peanut-specific IgE binding. An immunoblot strip containing the wild-type and mutated peptides of epitope 9 is shown in Fig. 1. Binding of pooled serum IgE to these individual peptides was dramatically reduced when either alanine or methionine was substituted for

each of the amino acids at positions 144, 145, and 147-150. Changes at positions 144, 145, 147, and 148 had the most dramatic effect when methionine was substituted for the wild type amino acid, resulting in less than 1% of peanut specific IgE binding to these peptides. In contrast, the substitution of an alanine for arginine at position 152 resulted in increased IgE binding. The remaining Ara h 1 epitopes were tested in the same manner and the intensity of IgE binding to each spot was determined as a percentage of IgE binding to the wild-type peptide (Table 1). Each epitope could be mutated to a non-IgE binding peptide by the substitution of a single alanine or methionine residue.

The amino acids within each epitope were classified according to whether they were hydrophobic (apolar), polar, or charged residues (Fig. 2). There were a total of 196 amino acids present in the 21 epitopes of Ara h 1 that were studied. Charged residues occurred most frequently (89/196), with hydrophobic residues (71/196) being the next frequent type of amino acid in the epitopes, and polar residues representing the least frequent amino acid group (36/196). Thirty-five percent of the mutated hydrophobic residues resulted in loss of IgE binding (<1% IgE binding), while only 25% and 17% of mutated polar and charged residues, respectively, had a similar effect. These results indicated that the hydrophobic amino acid residues within these IgE binding epitopes were the most sensitive to changes. In addition results from this analysis indicated that the amino acids located near the center of the epitope were more critical for IgE binding.

***Location of the IgE binding epitopes on the 3-D structure of* Ara h 1.** A homology-based model of Ara h 1 tertiary structure was generated to determine the location of the epitopes on this relatively large allergenic molecule. To construct this model, the primary amino acid

sequence of Ara h 1 was aligned to the highly homologous protein phaseolin, for which x-ray crystal structure data was available (Fig. 3). The quality of the Ara h 1 model was assessed using the protein health module of QUANTA and PROCHECK version 2.1.4 (28) from Oxford Molecular Inc. (Palo Alto, CA) and compared to the quality of the structures of phaseolin and canavalin[4] (21,22,29). Most of the backbone torsion angles for non-glycine residues lie within the allowed regions of the Ramachandran plot (Fig.4). Only 1.4% of the amino acids in the Ara h 1 model have torsion angles that are disallowed as compared to 0.3% and 0.6% of amino acids in phaseolin and canavalin, respectively (Table 2). In addition, the number of buried polar atoms, buried hydrophilic residues, and exposed hydrophobic residues in the Ara h 1 model are comparable to those found in the structures of phaseolin and canavalin (Table 2). Taken together these data indicate that the homology-based model of Ara h 1 tertiary structure is reasonable and similar to the structures of other homologous proteins that have been solved. The global fold of the Ara h 1 molecule and the position of epitopes 10-22 are shown in Fig. 5A. The tertiary structure of the molecule consists of two sets of opposing anti-parallel β-sheets in swiss roll topology joined by an inter-domain linker. The terminal regions of the molecule consist of α-helical bundles containing three helices each. Epitope 12 resides on an N-terminal α-helix while epitopes 20 and 21 are located on C-terminal α-helices. Epitopes 14, 15, and 18 are primarily β-strands on the inner faces of the domain and epitopes 16, 17, 19, and 22 are β-strands on the outer surface of the domain. The remainder of the epitopes are without a predominant type of higher secondary structure. A space filled model depicting the surface accessibility of the epitopes and critical amino acids is shown in Fig. 5B. Of the 35 residues

that affected IgE binding, 10 were buried beneath the surface of the molecule, and 25 were exposed on the surface.

Ara h 1 ***interacts with itself to form a stable trimeric structure.*** A rapid, reproducible fluorescence assay was developed in order to determine if the peanut allergen formed higher order structures similar to those observed for soybean vicilins. Purified, fluorescein labeled Ara h 1, 10 nM, was mixed with various concentrations of unlabeled Ara h 1. The anisotropy of fluorescence observed at each concentration was then determined and plotted as milli-anisotropy units (mP in arbitrary units) versus the concentration of Ara h 1 (Fig. 6). Measurement of fluorescence anisotropy reveals the average angular displacement of the fluorphor, which is dependent on the rate and extent of rotational diffusion. An increase in the size of the macromolecule through complex formation results in decreased rotational diffusion of the labeled species, which in turn results in an increase in anisotropy. The plateaus observed at protein concentrations between 0 and 20 nM and between 200 nM and 2 μM indicate the presence of a homogeneous species at these concentrations. The sharp increase in anisotropy observed at concentrations of Ara h 1 above 50 nM indicates that a highly cooperative interaction between Ara h 1 monomers had occurred that results in the formation of a stable homo-oligomeric structure. In order to determine the stoichiometry of this interaction, cross-linking experiments were performed, followed by SDS-PAGE analysis of the cross-linked products (Fig. 6, inset). Ara h 1 oligomers representing samples taken at the 200 nM concentration were subjected to limited chemical cross-linking with DSP. Cross-linked and non-cross-linked samples were resolved by SDS-PAGE and visualized by Coommassie staining of the gel. We found that limited cross-linking at 1 μM

DSP results in the formation of an electrophoretically stable complex with an apparent molecular mass of approximately 180 kDa, appropriate for an Ara h 1 trimer.

DISCUSSION

Food allergies are mediated through the interaction of IgE to specific proteins contained within the food. While the IgE binding epitopes from the major allergens of cow milk (30), codfish (31), hazel (32), soy (33) and shrimp (34) have all been elucidated there have been few, if any, characteristics found in common with these binding sites. Our work on the IgE binding epitopes of Ara h 1 also indicates that there is no common amino acid sequence motif found in all epitopes (10). However, we have determined that once an IgE binding site has been identified it is the hydrophobic amino acid residues that appear to play a critical role in immunoglobulin binding. The observation that alteration of a single amino acid leads to the loss of IgE binding in a population of peanut-sensitive individuals is significant because it suggests that while each patient may display a polyclonal IgE reaction to a particular allergen (10,11), IgE from different patients that recognize the same epitope must interact with that epitope in a similar fashion. Besides finding that many epitopes contained more than one residue critical for IgE binding, it was also determined that more than one residue type (ala or met) could be substituted at certain positions in an epitope with similar results. This allows for the design of a hypoallergenic protein that would be effective at blunting allergic reactions for a population of peanut sensitive individuals. Furthermore, a peanut where the IgE binding epitopes of the major allergens have been removed prevents the development of peanut hypersensitivity in individuals genetically predisposed to this food allergy.

The characteristics that have been attributed to allergenic proteins include their abundance in the food source, their resistance to food processing, and their stability to digestion by the gastrointestinal tract (14,15). The major peanut allergen, Ara h 1, has been shown to be an abundant protein (35) that survives intact most food processing methods (36), and is stable to digestion in *in vitro* systems designed to mimic the gastrointestinal tract (37). However, the physical characteristics that allow this protein to exhibit these properties have not previously been examined. Our observations on the tertiary structure of the Ara h 1 monomer and the determination that this protein readily forms a trimeric complex may help to determine why this protein is allergenic. For example, we have described the tertiary structure of the Ara h 1 protein as consisting of two sets of opposing anti-parallel β-sheets in swiss roll topology with the terminal regions of the molecule consisting of α-helical bundles containing three helices apiece. While there are numerous protease digestion sites throughout the length of this protein, the structure may be so compact that potential cleavage sites are inaccessible until the protein is denatured. In addition, the formation of a trimeric complex and further higher order aggregation may also afford the molecule some protection from protease digestion and denaturation and allow passage of Ara h 1 across the small intestine. It has been shown that some atopic individuals transfer more antigen across the small intestine in both the intact and partially degraded state (38). These physical attributes of the Ara h 1 molecule may help to explain the extreme allergenicity exhibited by this protein.

The only therapeutic option currently available for the prevention of a peanut hypersensitivity reaction is food avoidance. Unfortunately, for a ubiquitous food such as

peanut, the possibility of an inadvertent ingestion is great. This is complicated by the fact that all of the peanut allergens identified to date have sequence homology with proteins in other plants. This may explain the cross-reacting IgE antibodies to other legumes that are found in the sera of patients that manifest clinical symptoms to only one member of the legume family (39). The elucidation of the position of the Ara h 1 IgE binding epitopes clustered on the surface of the molecule enables us to better understand why these regions elicit the clinical symptoms associated with peanut hypersensitivity. Perhaps the presentation of multiple, clustered epitopes to mast cells results in a more efficient and dramatic release of mediators that result in the more severe clinical symptoms observed in patients with peanut hypersensitivity. We are currently exploring this possibility by comparing the IgE binding epitopes and tertiary structures of other legume allergens.

Finally, it has been suggested that an altered Ara h 1 gene could be developed to replace its allergenic homologue in the peanut genome, thus blunting allergic reactions in sensitive individuals who inadvertently ingest this food (10). Since the Ara h 1 gene product is such an abundant and integral seed storage protein, it would be necessary for the altered vicilin to retain as much of its native function, properties, and three-dimensional structure as possible. The data presented here indicate that development of a hypoallergenic vicilin is feasible. However, the effect of altering critical amino acids within each of the IgE binding epitopes has on the properties of this seed storage protein is currently unknown. Given the widespread use of peanuts in consumer foods and the potential risk this poses to individuals genetically pre-disposed to developing peanut allergy and to the health of individuals already peanut sensitive, this approach is currently being explored in our

laboratories.

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FOOTNOTES

1. The abbreviations used are: IgE, immunoglobulin E; DBPCFC, double-blind, placebo-controlled, food challenge; Fmoc, 9-fluorenylmethoxycarbonyl; TBS, tris-buffered saline; PBS, phosphate-buffered saline; DSP, dithiobis(succinimidyl propionate); PAGE, polyacrylamide gel electrophoresis.
2. The atomic coordinates for the crystal structure for this protein can be accessed through the Brookhaven Protein Data Bank under PDB #2PHL (12).
3. Maleki et al., manuscript in preparation.
4. The atomic coordinates for the crystal structure for this protein can be accessed through the Brookhaven Protein Data Bank under PDB #1CAU (29).
5. The amino acid sequence of this protein can be accessed through the GenBank database

under GenBank Accession Number L34402 (17).

FIGURE LEGENDS

Fig. 1. Single amino acid changes to epitope 9 result in loss of IgE binding to this epitope. Epitope 9 was synthesized with an alanine (*Panel A*) or methionine (*Panel B*) residue substituted for one of the amino acids and probed with a pool of serum IgE from 15 patients with documented peanut hypersensitivity. The letters across the top of each panel indicate the one-letter amino acid code for the residue normally at the position and the amino acid that was substituted for this residue. The numbers indicate the position of each residue in the Ara h 1 protein. WT, indicates the wild-type peptide (no amino acid substitutions).

Fig. 2. Hydrophobic amino acids are more critical to IgE binding. The type of each amino acid within the Ara h 1 epitopes was assessed relative to its importance to IgE binding. The closed boxes represent the total number of a particular type of amino acid residue found in all of the Ara h 1 epitopes. while the open boxes represent the number of that type of residue which, when replaced, was found to result in the loss of IgE binding.

Fig. 3. Alignment of the primary amino acid sequences and the α-carbon structural alignment of Ara h 1 and the phaseolin A chain. The upper panel represents the single letter amino acid code for Ara h 1 residues 172-586 (top line) and all of the phaseolin A chain (bottom line). The structurally conserved regions, shown in bold type. were used to develop the initial backbone of the Ara h 1 model. The other regions were used in protein loop searches to complete the tertiary structure of Ara h 1. Panel B represents the α-carbon alignment of the final model of Ara h 1 (white) vs the phaseolin A chain (yellow). Labeled

residues Asn1 and Arg415 represent the N- and C- terminus of the Ara h 1 model, respectively. Areas between labeled amino acids Asn169-Val193, Val212-Gly221, Phe240-Pro226, and Arg300-Asn323 represent areas of structural uncertainty due to insertions in Ara h 1 or unsolved sequences in phaseolin. Note that the residue numbers are shifted due to the N-terminal deletion from the Ara h 1 coding sequence found in the GenBank database[5].

Fig. 4. Most of the Phi/Psi torsion angles of the amino acid residues in the Ara h 1 tertairy structure model are allowed. A plot of the phi and psi angles for the amino acids in the Ara h 1 tertiary structure model is shown. Each dot within one of the boxes represents an amino acid that has acceptable torsion angles. Major outliers are indicated by their three letter amino acid code and position using the N-terminal as residue 1 as in figure 3.

Fig. 5. The majority of the Ara h 1 IgE binding epitopes are clustered in two regions of the allergen. The upper panel represents a ribbon diagram of Ara h 1 tertiary structure. The numbered red areas are IgE binding epitopes 10-22. Epitopes 13, and portions of 14, and 15 lie in an area of structural uncertainity. The lower panel is a space filling model of Ara h 1 tertiary structure. The red areas represent the IgE binding epitopes and the yellow atoms are the residues that were determined to be critical for IgE binding to occur.

Fig. 6. The Ara h 1 allergen forms a stable trimeric structure. Trace fluorescein labeled Ara h 1 was mixed with unlabeled Ara h 1 and fluorescence anisotropy measurements (mP) were made at each concentration. Each point represents the average of three different experiments. Samples from the 200 nM concentration were then subjected to cross-linking with varying concentrations of DSP and the products electrophoresed on SDS

polyacrylamide gels. Protein bands were visualized by Coommassie staining. Lower arrow indicates the Ara h 1 monomer (~60 kDa) and the upper band represents the Ara h 1 trimer (~180 kDa).

TABLE 1

Amino Acids Critical to IgE Binding

PEPTIDE	AA SEQUENCE	POSITION
1	AKS**SPY**Q**K**KT	25-34
2	QEP**DDL**KQKA	48-57
3	LE**YDP**RL**VY**D	65-74
4	GE**R**TR**GRQ**PG	89-98
5	PGDYDD**D**RRQ	97-106
6	PRREE**G**GRWG	107-116
7	REREED**W**R**Q**P	123-132
8	EDW**RRP**SHQQ	134-143
9	Q**PR**K**IR**PEGR	143-152
10	T**P**GQ**F**ED**FF**P	294-303
11	S**YL**Q**EF**SRNT	311-320
12	**F**NAE**F**NEIRR	325-334
13	EQEER**G**QRRW	344-353
14	DIT**NPI**N**L**RE	393-402
15	NNFGK**LF**EVK	409-418
17	**RRY**TARLKEG	498-507
18	EL**HL**L**GFG**IN	525-534
19	HRIFLAGD**K**D	539-548
20	IDQ**I**EKQ**A**K**D**	551-560
21	KDLA**FPG**SGE	559-568
22	KESHFV**S**ARP	578-587

Note. The Ara h 1 IgE binding epitopes are indicated as the single letter amino

acid code. The position of each peptide with respect to the Ara h 1 protein coding sequence is indicated in the right hand column. The amino acids that, when altered, lead to loss of IgE binding are shown as the bold, underlined residues. Epitopes 16 and 23 were not included in this study because they were recognized by a single patient who was no longer available to the study.

TABLE 2

Comparison of Amino Acid Torsion Angles and Buried and Exposed Residues from the Tertiary Structures of Ara h 1, Phaseolin, and Canavalin

	Ara h1	Phaseolin	Canavalin
Buried Polar Atoms	52	42	67
Buried Hydrophilic	16	7	10
Exposed Hydrophobic	2	2	3
Ramachandran Highly Favored*	309	280	250
Ramachandran Allowed*	56	40	71
Ramachandran Disallowed*	5	1	2

*Terminal amino acid residues, glycines, or prolines are not included in these categories.

TABLE 3

Alignment Of The Primary Amino Acid Sequences Of Ara h 1 And Phaseolin A Chain

```
NNPFYFPSRR FSTRYGNQNGRIRVLQRFDQRSRQFQNLQNHRIVQIEAKPNTLVLP  227
DNPFYFNSDNSWNTLFKNQYGHIRVLQRFDQQSKRLQNLEDYRLVEFRSKPETLLLP

KHADADNILVIQQGQATVTVANGN NRKSFNLDEGH     ALRIPSGFISYILNRH  278
QQADAELLLVVRSGSAILVLVKPDDRREYFFLTSDNPIFSDHQKIPAGTIFYLVNPD

DNQNLRVAKISMPVNTPGQFEDFFPASSRDQSSYLQGFSRNTLEAAFNAEFNEIRRV  335
PKEDLRIIQLAMPVNNPQIH EFFLSSTEAQQSYLQEFSKHILEASFNSKFEEINRV

LLEENAGGEQEERGQRRWSTRSSENNEGVIVKVSKEHVEELTKHAKSVSKKGSEEE  391
LFEEEGQQEGV                IVNIDSEQIKELSKHAKSSSRKSLSKQD

GDITNPINLREGEPDLSNNFGKLFEVKPDKKNPQLQDLDMMLTCVEIKEGALMLPHF  448
NTIGNEF              GNLTERTDNSLN       VLISSIEMEEGALFVPHY

NSKAMVIVVVNKGTGNLELVAVRKEQQQRGRREEEEDEDEEEEGSNREVRRYTARLK  505
YSKAIVILVVNEGEAHVELVGPKGNKETLEYE                  SYRAELS

EGDVFIMPAAHPVAINASSELHLLGFGINAENNHRIFLAGDKDNVIDQIE     KQ  557
KDDVFVIPAAYPVAIKATSNVNFTGFGINANNNNRNLLAGKTDNVISSIGRALDGKD

AKDLAFPGSGEQVEKLIKNQKESHFVSAR  586
VLGLTFSGSGDEVMKLINKQSGSYFVDAH
```

The Ara h 1 trimer is unstable at pH2.

In order to assess the stability of the Ara h 1 trimer at pHs that would be encountered in the human stomach, cross-linking experiments were performed using purified Ara h 1 protein suspended in a pH 2.1 buffer. Purified Ara h 1 (2 mM) was suspended in 500 μl of either a pH 2.1 buffer or a pH 7.6 buffer and allowed to incubate for one hour at room temperature. Cross-linking was performed using 5% DSP in DMF for varying lengths of time (10, 20, or 50 seconds). Results indicate that the Ara h 1 trimer is unstable at acidic pHs that would be encountered in the human stomach but that the monomer is stable at this pH. Further experiments indicate that the monomer is stable at pH 2.1 for greater than 8 hours at 37°C.

Figure 7. Portions of the Ara h 1 protein are resistant to chymotrypsin digestion.

Purified Ara h 1 (8.5 μM) was subjected to digestion with chymotrypsin (0.2 μM), at 37°C for varying lengths of time. Samples were withdrawn at various times and prepared for analysis. Panel A shows a coomassie stained protein gel. Panel B shows an immunoblot analysis using a pool of serum IgE from peanut sensitive patients. Panel C shows an immunoblot analysis using an antibody that recognizes a unique carbohydrate structure that includes a beta-1,2-linked xylose attached to the beta-linked mannose of the core oligosaccharide chain. Protease resistant Ara h 1 fragments were detected in all samples for up to 3 hrs after the addition of the enzyme. Many of the protease resistant fragments also bound serum IgE from peanut sensitive patients and were glycosylated.

Figure 8. Portions of the Ara h 1 protein are resistant to trypsin digestion.

Purified Ara h 1 (8.5 μM) was subjected to digestion with trypsin (0.01 μM) at 37°C for varying lengths of time. Samples were withdrawn at various times and prepared for analysis. Panel A shows a coomassie stained protein gel. Panel B shows an immunoblot analysis using a pool of serum IgE from peanut sensitive patients. Panel C shows an immunoblot analysis using an antibody that recognizes a unique carbohydrate structure that includes a beta-1,2-linked xylose attached to the beta-linked mannose of the core oligosaccharide chain. Protease resistant Ara h 1 fragments were detected in all samples for up to 3 hrs after the addition of the enzyme. Many of the protease resistant fragments also bound serum IgE from peanut sensitive patients and were glycosylated.

Figure 9. Portions of the Ara h 1 protein are resistant to pepsin digestion.

Purified Ara h 1 (8.5 μM) was subjected to digestion with pepsin (2 μg/ml) at 37°C for varying lengths of time. Samples were withdrawn at various times and prepared for analysis. Panel A shows a coomassie stained protein gel. Panel B shows an immunoblot analysis using a pool of serum IgE from peanut sensitive patients. Protease resistant Ara h 1 fragments were detected in all samples for up to 3 hrs after the addition of the enzyme. Many of the protease resistant fragments also bound serum IgE from peanut sensitive patients and were glycosylated (not shown).

Figure 10. Non-allergenic proteins are sensitive to protease digestion.

Several non-allergenic proteins were subjected to digestion with trypsin and chymotrypsin under the same conditions as described in Figs. 4 and 5. The results from a representative protein (myo D, a mammalian transcription factor) are shown. In contrast to Ara h 1, myo D did not produce long-lived protease resistant fragments.

Summary

The Ara h 1 trimer, which is stable at high salt concentrations, is unstable at an acidic pH (2.1) that is found in the human stomach.

The Ara h 1 monomer is stable at pH 2 and portions of this allergen are resistant to digestion with pepsin, trypsin, and chymotrypsin.

Although the Ara h 1 protein contains numerous protease cleavage sites, located in almost all of the IgE binding epitopes, proteolytic digestion results in several large resistant fragments.

Ara h 1 peptides that are resistant to protease digestion contain IgE binding epitopes.

Most of the protease-resistant Ara h 1 peptides contain a β1,2-linked xylose attached to the β-linked mannose of the core oligosaccharide chain.

Conclusions

As reported by other labs, allergens are resistant to digestive enzymes whereas non-allergenic proteins are quickly degraded. However, in contrast to other reports, our results indicate that peptide fragments, not the entire protein, survive for significant periods of time in the presence of proteases. In addition, these protease resistant fragments contain IgE binding sites and carbohydrate. These studies indicate that long-lived protease-resistant protein fragments may play a role in the etiology of food allergic disease because of their ability to survive the human gastrointestinal tract long enough to gain access to the immune system.

Glycinin, A Third Major Peanut Allergen Identified By Soy-Adsorbed Serum IgE from Peanut Sensitive Individuals. *P Rabjohn, CM West, E Helm, R Helm, JS Stanley, SK Huang, H Sampson, AW Burks, and GA Bannon*. University of Arkansas Medical School, Little Rock, AR, Johns Hopkins University, Baltimore, MD, Mt. Sinai Hospital, New York, NY.

Two major allergens involved in peanut hypersensitivity, Ara h 1 and Ara h 2, have been isolated and extensively characterized. These allergens were identified on the basis of their ability to bind serum IgE from >90% of peanut hypersensitive patients. A third peanut allergen , Ara h 3 (~14 kD), was identified by using soy adsorbed serum IgE antibodies from peanut sensitive individuals. Amino terminal sequencing revealed that the first 23 amino acids of this protein had significant sequence homology with the glycinin family of seed storage proteins. A full length cDNA clone was isolated from a peanut cDNA library using oligonucleotides derived from this amino acid sequence. This clone encoded a 510 amino acid long protein with high homology to the glycinins. Further analysis of the immunoblot data and the Ara h 3 clone indicated that the 14 kD protein identified by soy adsorbed serum IgE represented the amino terminal end of a proteolytically cleaved 60 kD glycinin protein. The major linear IgE binding epitopes of this entire allergen were mapped by probing overlapping peptides with pooled serum IgE from 11 peanut sensitive patients. Ten IgE binding regions were identified, distributed throughout the length of the Ara h 3 protein. In an effort to determine the 6-10 amino acid long epitopes, overlapping 10mers will be made through each region with pooled positive serum. Once these are identified a set of all epitopes will be made and probed with individual serum IgE. In this manner the major IgE binding epitopes and prevalence of recognition by serum IgE within the peanut sensitive population will be determined.

INTRODUCTION

Approximately 1-3% of the USA population suffers from some form of food allergy. Peanuts, tree nuts, and shellfish are responsible for the majority of food hypersensitivity reactions in adults, while peanuts, milk, and eggs account for the majority of reactions in children. The reaction to peanut is more severe than the reaction to other foods, often resulting in fatal anaphylaxis. While most children outgrow allergies to milk and eggs, peanut allergies persist into adulthood, lasting the entire lifetime of the individual. Currently, avoidance is the only treatment for patients with peanut allergies; however, the inclusion of peanut as a protein extender in processed foods makes accidental consumption almost inevitable. Despite the prevalence of peanut hypersensitivity in children, coupled with an increasing number of deaths each year from peanut-induced anaphylaxis, the identification of unique, clinically-relevant allergens from peanut is incomplete, limiting our understanding of their role in the immunobiology of hypersensitivity reactions.

Devising better methods for managing peanut hypersensitivity requires a thorough understanding of how allergens and the immune system interact. The first step is understanding this interaction is to identify and characterize regions of the allergen that are initially recognized by the immune system. Ara h 3 is one of the three major allergens from peanut being characterized by our laboratory at the protein level. The cDNA encoding Ara h 3 has been cloned, sequenced, and expressed in a bacterial system. The derived amino acid sequence has been used to construct synthetic peptides so that the linear, IgE-binding regions within the primary sequence can be identified.

The goal of this research is to identify the linear, IgE-binding epitopes of Ara h 3. By identifying critical residues required for IgE-binding within each epitope, it will be possible to mutagenize the Ara h 3 cDNA to encode a protein that escapes IgG recognition.

FIGURE 11

Ara h 3 Amino Acid Sequence

A third peanut allergen, Ara h 3, was originally identified as a 14 kD protein by soy-adsorbed IgE sera from peanut hypersensitive individuals. Amino-terminal sequencing revealed that the first twenty-three amino acids (indicated in boldface) showed sequence similarity to the glycinin family of seed-storage proteins. A cDNA clone encoding 510 amino acids was isolated from a mature peanut expression library using oligonucleotides derived from the amino-terminus of the protein. Further sequence analysis revealed that the 14 kD protein originally identified by soy-adsorbed sera represents the amino-terminal portion of a 60 kD preproglobulin.

FIGURE 12

Sequence Comparison of Ara h 3 with Related Legume Glycinins

Ara h 3 showed 62-72% sequence identity with other legume glycinins. Sequences were aligned using the Wisconsin GCG analysis program. G1 Soy is the glycinin G1 precursor containing the A1a-Bx chains (*Glycine max*, GenBank P04776), G2 Soy is the glycinin G2 precursor containing the A2-B1a chains (*Glycine max*, GenBank A91341), and A2 Pea is the legumin A2 precursor (*Pisum sativum*, GenBank X17193). (A) Conserved region near the amino-terminus of the acidic chain. Shaded residues represent glycinin signature sequence. (B) Conserved region near the amino-terminus of the basic chain. Shaded residues represent glycinin signature sequence. The *Ara h 3* primary sequence contains 24 or the 26 invariable residues corresponding to glycinin signature sequence.

FIGURE 13

Serum IgE From a Pool of Peanut Hypersensitive Patients Recognizes Recombinant Ara h 3

(A) The cDNA encoding Ara h 3 was cloned into the pET 24(b)+ plasmid. This plasmid allows bacterial expression of the recombinant protein with a 14-amino acid polypeptide tail containing a polyhistidine tag at the carboxy-terminus. The molecular weight standards, MW, are expressed in kilodaltons (kD). Lane A, 4h induction of pET 24 harboring no insert; lane B, uninduced Ara h 3; lane C, 1h induction of Ara h 3; lane D, 2h induction; lane E, 3h induction; lane F, 4h induction; lane G is blank; lane H, native Ara h 1 (63.5 kD). (B) Immunological detection was performed using a pool of serum IgE from peanut hypersensitive patients.

FIGURE 14

Identification of a Core IgE-Binding Epitope on the Ara h 3 Allergen.

(A) Detailed epitope mapping was performed on IgE-binding regions listed in Table 1 by synthesizing overlapping peptides ten amino acids in length offset from the previous peptide by two residues. These peptides were probed with a pool of serum IgE from 11 patients with peanut hypersensitivity. The data shown represents amino acids 372-395. (*B*) The amino acid sequence (residues 372-395) of Ara h 3 that was tested in *A* is shown. Residues in boldface correspond to common IgE-binding amino acids of the spots shown in *A*.

FIGURE 15

Identification of the Immunodominant Epitopes of Ara h 3

(A) IgE-binding peptides (1-4) were synthesized and probed individually with serum IgE from 20 peanut-hypersensitive patients. Numbers across the top represent individual patients, nine of which are shown here. (B) The percentage of individual peanut-hypersensitive patients recognizing epitopes (1-4) ranges from 5% to 100%. The IgE-binding sequence and its corresponding position in the primary sequence of Ara h 3 is also shown.

FIGURE 16

Single Amino Acid Changes to Peptide 2 Result in Loss of IgE Binding to This Epitope

Peptide 2 was synthesized with an alanine residue (except position 254 which was substituted with leucine) substituted for one of the amino acids at each position in the peptide. The synthesized peptides were probed with a pool of serum IgE from peanut hypersensitive patients whose IgE has previously been shown to recognize this peptide. The letters across the top of each panel indicate the one-letter amino acid code for the residue normally at that position and the amino acid substituted for that residue. The numbers indicate the position of each residue in the Ara h 3 protein. *WT* indicates the wild-type peptide with no amino acid substitutions.

TABLE 4

Multiple IgE-binding Regions Identified on the Ara h 3 Allergen

Epitope mapping was performed on the Ara h 3 allergen by synthesizing the entire protein in 15 amino acid-long overlapping peptides offset from the previous peptide by seven residues. 71 overlapping peptides were synthesized to determine which regions in the primary sequence were recognized by serum IgE from peanut hypersensitive patients. In this manner, the entire length of the Ara h 3 allergen could be studied in short, overlapping fragments.

Region	Peptide	Sequence
1	26	FNLAGNTEQEFLRYQ
	27	EQEFLRYQQQSRQSR
	28	QQQSRQSRRRSLPYS
2	34	GQEEENEGGNIFSGF
	35	GGNIFSGFTPEFLEQ
	36	FTPEFLEQAFQVDDR
3	43	RADEEEEYDEDEYEY
	44	YDEDEYEYDEEDRRR
	45	YDEEDRRRGRGSRGR
4	54	LSAEYGNLYRNALFV
	55	LYRNALFVAHYNTNA
	56	VAHYNTNAHSIIYRL

SUMMARY

- The cDNA clone encoding Ara h 3, a third major peanut allergen was cloned from a peanut expression library

- Ara h 3 is a member of the glycinin family of seed-storage proteins

- Recombinant Ara h 3 has been expressed in a bacterial system and is recognized by IgE from peanut-hypersensitive patients

- Linear, B-cell epitopes have been identified within the primary sequence of Ara h 3

- The percentage of patients recognizing each epitope on Ara h 3 has been determined

- Mutation of critical residues within the Ara h 3 epitopes reduces and eliminates IgE-binding

Rapid Isolation Of Peanut Allergens And Their Physical Chemical And Biological Characterization. *R Kopper, S Maleki, R Helm, H Sampson, SK Huang, G Cockrell, AW Burks, and GA Bannon.* University of Arkansas Medical School, Little Rock, AR, Johns Hopkins University, Baltimore, MD, and Mt. Sinai Hospital, New York, NY.

The investigation of IgE-mediated peanut hypersensitivity requires the identification and characterization of the allergens involved in this immunologic disorder. A rapid, procedure has been developed for the extraction of large quantities (95% pure) of the major peanut allergens, Ara h 1 and Ara h 2. The purified allergens were analyzed by circular dichroism spectroscopy and found to have alpha helix and beta sheet domains that could be unfolded under increasingly denaturing conditions. Ara h 1 was shown to be very stable and showed minor changes in its secondary structure up to 5 M urea, whereas Ara h 2 was denatured at much lower concentrations. Using fluorescence polarization spectroscopy and cross-linking, fluorescein-labeled Ara h 1 was shown to self-associate in a highly cooperative manner into distinct homo- trimers in the presence of high salt concentrations. These results indicate that the interface is stabilized primarily by hydrophobic interactions. This higher order structure bound IgE from peanut sensitive individuals indicating that the epitopes are exposed on the molecule's surface. A decrease in the slope of the Ara h 1 titration curves in the presence of increasing concentrations of salt demonstrates the involvement of ionic interactions in the cooperative association of the trimers. Current experiments are underway to determine if this multimeric Ara h 1 molecule forms at pH ranges that would be encountered in the digestive tract and whether this molecule is resistant to digestion by proteases.

INTRODUCTION

Every year twenty million Americans suffer from one or more types of allergies. Food allergies occur in 6-8% of children and 1-2% of adults. Peanut, egg and milk are responsible for 80% of food allergies that occur in children. Peanut allergy is a life-threatening, lifelong disorder. The only current method for prevention of food allergy is avoidance. Children become tolerant to most food allergies with age (e.g. eggs, milk, shellfish, soy, etc.), however peanut allergy is rarely outgrown. Thus, it is increasingly difficult for an allergic individual to avoid an abundantly utilized and most often disguised food source such as peanuts, leading to accidental ingestion, anaphylaxis and possibly death. To understand IgE–mediated food hypersensitivity reactions it is important to identify and characterize the allergens that are responsible for the immunologic disorder. It is also necessary to understand the basic molecular interactions involved in allergen recognition by the immune system.

In the current work we have carried out rapid, large scale purification and biophysical characterization of two major peanut allergens Ara h1 and Ara h 2. The purification of these native peanut proteins has not only enabled us to study the response of peanut specific T-cell lines and mast cells to treatment with Ara h 1 and Ara h 2 independent of other peanut proteins but, also allowed the establishment of Ara h 1 and 2 specific T-cell clones. Detailed biochemical and biophysical characterization of these proteins have also been carried out utilizing fluorescence polarization, western blot analysis and cross-linking.

Table 5. Summary of K_{app} and ρ-values for Ara h 1 oligomer formation.

Ara h 1 Oligomer	Salt concentration (mM)	K_{app} (μM)	ρ-value (coop.)
monomer to trimer	0	*	*
monomer to trimer	100	0.065	2.4
monomer to trimer	300	0.07	2.25
monomer to trimer	500	0.095	2.1
monomer to trimer	900	0.12	2.1
monomer to trimer	1400	0.17	2.2
monomer to trimer	1800	0.17	2.1
trimer to hexamer	100	32.6	1.1
trimer to hexamer	400	36	1.03
trimer to hexamer	600	41	1.13
trimer to hexamer	800	45	1.0
trimer to hexamer	1100	48	0.9
trimer to hexamer	1300	54	0.9
trimer to hexamer	1800	65	0.8

* These values cannot be determined by the fitting program used.

CONCLUSIONS

1. Large milligram quantities of highly purified protein allergens can be obtained in one days time and utilized for various applications such as:

   a- preparation of highly pure, standardized allergens for use as diagnostic tools such as skin prick tests.

   b- highly quantitative biochemical and biophysical characterizations of the allergens.

   c- to establish allergen specific T-cell lines, study the effect of allergens on mast cells and other cell types and for comparison to other allergens and their effects on the immune system.

2. Purified proteins were recognized by the components of the immune system that are found to play important roles in allergy and demonstrate biological activity associated with their allergenicity, i.e.:

   a- native Ara h 1 and Ara h 2 were both recognized by serum IgE of allergic individuals.

   b- Ara h 1 and Ara h 2 were able to stimulate T-cell proliferation and mast cell degranulation independently of other peanut proteins.

3. The biophysical characteristics of Ara h 1 and Ara h 2 may have strong implications for their allergenicity:

   a- Ara h 1 forms highly stable trimeric and hexameric oligomers primarily shown to be stabilized through hydrophobic interactions. Formation of these highly stable oligomers may be important in it's protection against digestion.

   b- Ara h 2 does not form homo-oligomeric structures, but has been shown to have a low affinity interaction with trypsin. Trypsin binding ability of Ara h 2 may be involved in it's role as a potent allergen.

Fig. 23. SDS-PAGE analysis at various stages of allergen purification and immunoblot using serum IgE from allergic individuals. Panels A is the protein profiles resulting from the purification of Ara h 2. **Panel B** is the protein profiles resulting from the purification of Ara h 2 that was blotted to nitrocellulose and detected by western blot analysis using serum IgE from allergic individuals as the primary antibody. Lanes are as follows: *lanes A1 and B1*, crude peanut extract; lane *A2* and B2, 25% ammonium sulfate pellet; *A3* and B3, Ara h 2 fraction following anion exchange chromatography; *lane A4 and B4*, Ara h 2 fraction following hydrophobic chromatography.

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Fig. 24 Molecular mechanisms involved in peanut-stimulated T-cell activation. T cells lines derived from peanut sensitive individuals were stimulated with CPE and then at various times after stimulation (0, 5, and 10 minutes) the cells were suspended in lysis buffer (1% NP-40, 0.5 % Na-deoxycholate, 0.1% SDS, 1 mM EDTA, 1 mM $MgCl_2$, 50 mM NaF, 20 mM -glycero-phosphate, 1 mM Na-ortho-vanadate, 1 mM PMSF, 10 µg/ml Aprotinin, 10 µg/ ml Leupeptin, 10 µM Pepstatin in PBS, pH 7.5) and homogenized until they were lysed. Immunoprecipitations were performed by using polyclonal rabbit anti-ZAP-70 antibody directly conjugated to agarose beads (Zap-70-LR, Santa Cruz Biotechnology, Inc., Santa Cruz, CA). The immunoprecipitated pellet was then resuspended in SDS-sample buffer and electrophoresed on 12% polyacrylamide gels. SDS-PAGE resolved proteins were then transferred to nitrocellulose electrophoretically. The membranes were washed, blocked, and probed for co-immunoprecipitated and phosphoryoated proteins using standard western blot techniques with the appropriate primary antibodies: (**A**) mouse anti-T-cell receptor-ζ chain (Zymed Laboratories Inc., San Francisco, Ca), (**B**) anti-phospho-tyrosine, (**C**) anti-ERK I antibody or (**D**) PY-20 (from Santa Cruz Biotechnology, Inc., Santa Cruz, CA.). The membrane was then incubated in the presence of appropriate secondary antibody (either anti-rabbit or anti-mouse IgG) conjugated to Horse Radish peroxidase enzyme (HRP) and the signal detected using ECL western blotting reagents (Amersham International plc, Buckinghamshire, England.

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Fig. 55 Proliferation of T cells isolated from peanut allergic individuals. The peripheral blood lymphocytes (PBLs) of 12 peanut sensitive individuals and 5 non-allergic individuals (data not shown) were isolated from whole blood using ficoll hypaque. Cells were washed and suspended in media at the concentration of 4 x 10^6 cells/ml. Three aliquots (1 ml each) were placed in 24 well tissue culture plates and stimulated with 50 µg/ml crude peanut extract (CPE) every 10-14 days in order to establish peanut specific T-cell lines. For the T-cell proliferation assay, 12 wells of a 96 well plate at 2 x 10^5 PBLs/well were stimulated with media (control), crude peanut extracts (CPE, 50 µg/ml), wild type Ara h 2 (10 µg/ml), or crude rice extracts (Rice, 50 µg/ml, as negative control) at 37^{O}C. T-cell proliferation was estimated by quantitating the amount of ^{3}H-thymidine incorporation into the DNA of proliferating cells. ^{3}H-thymidine incorporation is reported as stimulation (SI) above media treated control cells. The graph represents the proliferation of T cells (**y-axis, SI**) from each individual plotted versus stimulant (**x-axis**).

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Fig. 56 The $CD4^+$ and $CD8^+$ profiles of the T-cell lines of peanut allergic individuals. T cells were stained with FITC-labeled anti-CD4 and PE-labeled anti-CD8 antibodies in order to determine the phenotype of the peanut specific T-cell lines established. FACS analysis was used to determine the percent of $CD4^+$ and $CD8^+$ cells in the peanut specific T-cell lines utilized in Ara h 2 signal transduction and epitope mapping studies. The percentages determined (**y-axis**) were plotted versus the initials of the individual patients (**x-axis**)used to establish these cell lines. This panel represents the CD4/CD8 profiles of T-cell lines established from allergic individuals.

Fig. 25 Synthetic overlapping peptides of Ara h 2. In order to determine the T-cell epitopes of peanut allergen Ara h 2, 29 different peptides representing the entire protein were synthesized. Each peptide was 20 amino acids long and was offset from the previous peptide by 5 amino acids. In this manner we were able to cover the entire protein sequence by overlapping peptides. The primary amino acid sequence of the Ara h 2 protein represented as the one letter amino acid code and individual peptides of Ara h 2 that were used to map T-cell epitopes are shown.

Fig. 26 Proliferation of T cells isolated from peanut allergic and non-peanut allergic individuals in response to Ara h 2 derived peptides. T cells were isolated from 17 peanut allergic individuals and 5 non-peanut allergic individuals (not shown) and placed into 96 well plates at 4 x 10^4 cells/well and treated in triplicates with media or Ara h 2 peptides (10mg/ml). The cells were allowed to proliferate for 6 days and then incubated with ^{3}H-thymidine (1mCi/well) at 37 °C for 6-8 hrs and then harvested onto glass fiber filters. T-cell proliferation was estimated by quantitating the amount of ^{3}H-thymidine incorporation into the DNA of proliferating cells. ^{3}H-thymidine incorporation is reported as stimulation (SI) above media treated control cells. Each graph represents the proliferation of T cells (**x-axis**) from each individual plotted versus the 29 overlapping peptides (**y-axis**) spanning the entire Ara h 2 protein from the amino-(peptide 904) to carboxyl terminus (peptide 932).

Fig.27 Identification of the Ara h 2 peptides that caused T-cell proliferation in the majority of patients tested. All of the data in Figure 2 was compiled and plotted as a stimulation index versus the Ara h 2 peptides. The mean proliferation and standard error of 17 peanut allergic individuals and 5 non-allergic individuals (data not shown) were calculated and plotted as mean stimulation index of atopic individuals versus the 29 overlapping peptides spanning the entire Ara h 2 protein from the amino-(peptide 904) to carboxyl terminus (peptide 932).

T-Cell Responses In Food Allergy: Identification Of T-Cell Epitopes On A Major Peanut Allergen.

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The development of an IgE response to an allergen involves a series of interactions between antigen-presenting cells (APCs), T cells, and B cells. The critical role T-cells play in the production of allergen-specific IgE has been studied in a variety of aeroallergens. However, the role of T lymphocytes and antigen specificity in the induction and regulation of the food allergic response is less well defined. Ara h 2 is one of the major allergens in peanut shown to stimulate IgE mediated disease in humans. We have used overlapping synthetic peptides spanning the entire protein to determine the T cell epitopes of Ara h 2. Peanut specific T cell lines were established from the peripheral blood of 12 atopic patients and 4 nonatopic controls. All of the cell lines were shown to consist of predominantly CD4+ T cells. The proliferation of the T cells in response to the 29 individual peptides was measured. Four immunodominant T cell epitopes were identified for Ara h 2, epitope 1 (AA 18-28), epitope 2 (AA 45-55), epitope 3 (AA 95-108), and epitope 4 (AA 134-144). Epitope 1, 2, and 4 have overlapping sequences with Ara h 2 B cell epitopes whereas, epitope 3 does not overlap IgE binding epitopes therefore providing a possibility for the development of a non-anaphylactic, T cell directed, immunotherapeutic peptide.

INTRODUCTION

Approximately 8% of children and 1-2% of adults have some type of food allergy. Peanuts, fish, tree nuts, and shellfish account for the majority of food hypersensitivity reactions in adults, while peanuts, milk, and eggs cause over 80% of food hypersensitivity reactions in children. Unlike the food hypersensitivity reactions to milk and eggs, peanut hypersensitivity reactions usually persist into adulthood and last for a lifetime. In addition, hypersensitivity reactions to peanuts tend to be more severe than those to other food allergens. Several reports have detailed fatal and near-fatal anaphylactic reactions occurring in adolescents and adults following the ingestion of peanuts or peanut products.

Currently, the only effective treatment for patients with peanut hypersensitivity is avoidance of any food products which contain the allergen. This is becoming more difficult due to the inclusion of peanuts and peanut products as protein extenders in many different foods. Potential immunotherapeutic approaches to food hypersensitivity might include the competitive inhibition of allergen presentation with antibodies specific to the TCR V region or with soluble TCRs that would bind epitopes preventing them from binding to cell associated receptors. Another avenue of approach might be the modulation of Th cell development to favor Th_1 cytokine responses by alteration of T-cell epitope structure. All of these approaches require in-depth knowledge of the T-cell epitope structure and T-cell signaling pathways that are activated by allergen-specific interaction with receptors. We report complete T-cell epitope mapping of Ara h 2, a major peanut allergen. Peanut specific T-cell lines were established and used to map the immunodominant epitopes of Ara h 2, determine the surface receptor and cytokine expression profiles. T-cell epitopes have been compared to previously mapped B-cell epitopes of Ara h 2.

Fig. 25 . **Peanut allergen Ara h 2.**

In order to determine the T-cell epitopes of peanut allergen Ara h 2, 29 different peptides representing the entire protein were synthesized. Each peptide was 20 amino acids long and was offset from the previous peptide by 5 amino acids. In this manner we were able to cover the entire protein sequence by overlapping peptides. The primary amino acid sequence of the Ara h 2 protein represented as the one letter amino acid code and individual peptides of Ara h 2 that were used to map T-cell epitopes are shown.

Fig. 26 . **Proliferation of T cells isolated from peanut allergic and non-peanut allergic individuals in response to Ara h 2 derived peptides.**

T cells were isolated from 17 peanut allergic individuals and 5 non-peanut allergic individuals (not shown) and placed into 96 well plates at 4 x 10^4 cells/well and treated in triplicates with media or Ara h 2 peptides (10μg/ml). The cells were allowed to proliferate for 6 days and then incubated with ^{3}H-thymidine (1μCi/well) at 37 C for 6-8 hrs and then harvested onto glass fiber filters. T-cell proliferation was estimated by quantitating the amount of ^{3}H-thymidine incorporation into the DNA of proliferating cells. ^{3}H-thymidine incorporation is reported as stimulation (SI) above media treated control cells. Each graph represents the proliferation of T cells (x-axis) from each individual plotted versus the 29 overlapping peptides (y-axis) spanning the entire Ara h 2 protein from the amino-(peptide 904) to carboxyl terminus (peptide 932).

Fig. 27 . **Identification of the Ara h 2 peptides that caused T-cell proliferation in the majority of patients tested.**

All of the data in Figure 2 was compiled and plotted as a stimulation index versus the Ara h 2 peptides. The mean proliferation and standard error of (panel A) 17 peanut allergic individuals and (panel B) 5 non-allergic individuals were calculated and plotted as mean stimulation index of atopic individuals versus the 29 overlapping peptides spanning the entire Ara h 2 protein from the amino-(peptide 904) to carboxyl terminus (peptide 932).

Fig. 28 The $CD4^+$ and $CD8^+$ profiles of the T-cell lines of peanut allergic individuals.

T cells were stained with FITC-labeled anti-CD4 and FITC-labeled anti-CD8 antibodies in order to determine the phenotype of the peanut specific T-cell lines established. FACS analysis was used to determine the percent of $CD4^+$ and $CD8^+$ cells in the peanut specific T-cell lines utilized in Ara h 2 epitope mapping and plotted versus the initials of the individual patients used to establish these cell lines. Panel A represents the CD4/CD8 profiles of T-cell lines established from allergic individuals while panel B represents the CD4/CD8 profiles of T-cell lines established from non-allergic individuals.

Fig. 29 The IL-4 secretion profiles of a representative sample of T cells.

The supernatant was collected from T-cells stimulated with immunodominant peptides and an ELISA assay was utilized to measure IL-4 concentrations in the media. IL-4 concentration is plotted versus the 29 overlapping peptides spanning the entire Ara h 2 protein from amino- (peptide 904) to carboxyl terminus (peptide 932).

Fig. 30. Comparison of the T-cell and B-cell epitopes of Ara h 2.

The primary amino acid sequence of the Ara h 2 protein is represented as the one letter amino acid code. The T-cell epitopes of Ara h 2 that have been identified in this study are depicted as bold, italicized letters and the immunodominant B-cell epitopes determined in previous work are underlined. In general, the IgE binding epitopes do not overlap with the T-cell epitopes.

CONCLUSIONS

1. Four immunodominant T-cell epitopes have been identified for Ara h 2 using T cells isolated from 17 atopic individuals: peptides 907-908 (epitope 1), 911-914 (epitope 2), 923-926 (epitope 3) and 930-932 (epitope 4).

2. Similar T-cell epitopes were identified for Ara h 2 using T cells isolated from 5 non-atopic individuals.

3. T-cell lines established from both atopic and non-atopic individuals were primarily $CD4^+$.

4. T-cells from both atopic and non-atopic individuals seemed to secrete IL4 in response to treatment with immunodominant peptides. However, T-cells of non- atopic individuals seemed to secrete more IL4 in response to epitope 2 than epitope 1.

5. On average T-cells of the non-atopic individuals secreted lower levels of IL4 than the T-cells of atopic individuals.

6. When comparing the B-cell (previously described) and T-cell epitopes it is evident that the immunodominant epitopes do not overlap to any significant extent. This is very important in the development of peptide mediated immunotherapies towards modulating Th cell development to favor Th_1 type cytokine responses.

Tertiary Structure Of The Major Peanut Allergen Ara h 1: Implications For The Bioengineering Of A Hypoallergenic Protein.

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Allergy to peanut is a significant health problem because of the prevalence and potential severity of the reaction. Ara h 1, a major peanut allergen, has been isolated and characterized and was shown to consist of 626 amino acids and contain 23 linear IgE-binding epitopes, 6-10 residues in length. The amino acids important for peanut-specific IgE binding were determined by synthesizing wild type and mutant peptides with single alanine, glycine or methionine substitutions at each position followed by incubation in pooled serum from patients with peanut hypersensitivity. From this analysis it was determined that amino acids which reside in the middle of the epitope were generally more critical for IgE binding. Furthermore, though polar charged residues occur most frequently within the epitopes, apolar residues were found to be more important for IgE binding. In addition, it was found that each epitope could be mutated resulting in loss of ability to bind IgE with only a single amino acid substitution. To further characterize the epitopes a homology-based molecular model of the Ara H 1 protein was made. The model represents residues 171-586 allowing visualization of epitopes 10-22. The majority of these epitopes appear to be clustered to certain areas of the molecule. Many of the critical amino acids involved in binding are evenly distributed on the surface and not buried in the hydrophobic core. The information from the mutational analysis along with the molecular model will aid in the design of immunotherapies and enhance the ability to design functional hypoallergenic proteins.

INTRODUCTION

Peanut allergy is a significant health problem because of its high prevalence and the potential severity of the reaction (fatal anaphylaxis). The reaction involves the binding of allergenic proteins to IgE bound to mast cells. This induces a signal transduction cascade that ultimately leads to the release of the mediators that give rise to the clinical symptoms. Currently, the only effective treatment for food allergy is avoidance of the food.

The Ara h 1 protein from peanut has been shown to be recognized by serum IgE from >90% of peanut sensitive individuals, indicating that it is a major allergen involved in the clinical etiology of this disease. One of the current options being explored to decrease the risk of allergic reaction is the production of hypoallergenic proteins by alteration of the IgE epitopes. However, this strategy is difficult in the case of Ara h 1 because this particular protein is one of the most abundant in the seed and has been shown to contain 23 epitopes. To assess the ability to produce a hypoallergenic Ara h 1 protein, a molecular model of this 63 kDa allergen and the amino acids important for peanut-specific IgE-binding were determined in an effort to more clearly define the epitope regions and target amino acids for mutation.

The model of Ara h 1represents the conserved region between the vicilin family of proteins allowing visualization of epitopes 10-22. The epitopes are located primarily in two areas of the molecule, despite even distribution in the primary amino acid sequence. The epitopes also share no tertiary structure motifs and some lie within the interior of the molecule. To determine the amino acids critical for IgE binding within each epitope, wild type and single point mutation epitope peptides were constructed and tested for binding using pooled polyclonal IgE from sera obtained from 15 peanut-allergic patients. From this analysis it was determined that hydrophobic residues were found to be most important for IgE binding, even though polar charged residues occur more frequently. More importantly, there was at least one amino acid in each epitope that when changed resulted in a significant loss of IgE binding. Critical amino acids were mutated within the molecular model and analyzed to establish the potential of changing these residues in terms of stability of the tertiary structure. The results indicate that it may be possible to produce a hypoallergenic Ara h 1 protein through the replacement of single amino acids in the IgE binding epitopes.

SUMMARY

1. **Twenty one epitopes of Ara h 1 can be altered to a non-IgE binding peptide by a single amino acid substitution with either alanine or methionine.**

2. **Hydrophobic residues located in the middle of the epitopes are more critical for IgE binding.**

3. **The epitopes are clustered in two regions of the Ara h 1 monomer.**

4. **These studies will aid in the development of a hypoallergenic clone that is stable and retains its functional properties.**

IgE-Binding of Homologous Legume Vicilins and Glycinins of Soybean and Peanut Allergens. RM Helm, G Cockrell, SJ Stanley, HA Sampson, GA Bannon, AW Burks. University Arkansas for Medical Sciences, Little Rock, AR; Mt. Snai Hospital, New York, NY.

Allergic reactions to soybeans, compared to fish and peanuts, are unique in that the clinical reaction id typically outgrown in the first 3-5 years of life. We are using amino acid homology -based data searches, peanut-specific, and soy-specific serum to screen allergens from soybeans to identify and characterize differences in peanut and soybean vicilin and glycinin seed storage proteins. A GenBank search for amino acid homology to Ara h 1 identified a 47% amino acid sequence homology to soybean β-conglycinin. To determine the specific amino acid regions that bind peanut-and soy-specific IgE to soybean β-conglycinin, we used the SPOTS method to prepare 15-mer peptides overlapped by 8 amino acids for the entire amino acid sequence. Our results identified 4 common IgE-binding regions; however, there were 2 unique soybean positive IgE-binding regions (amino acid sequences 265-279 and 245-269) and 5 unique peanut positive IgE binding regions (amino acid sequences 50-63, 65-78, 376-399, 4110425, and 586-600). Using prep cell and 2-D SDS-PAGE, and serum from soy-sensitive individuals, a 20-22 kD allergen was identified using Western IgE, immunoblot analysis. N-terminal sequencing revealed this protein to be the soybean glycinin subunit, A2B1a. The B1a region of this subunit showed approximately 60% homology to a portion to a recently identified peanut allergen, Ara h 3. We are using these combined methods to investigate the epitope specificity; that may account for the different clinical spectra evident in these two legume-induced immediate type hypersensitivities.

INTRODUCTION

Legume grains such as peanuts and soybeans are being increasingly used as sources of protein for humans, a phenomenon that may be responsible for an increased rise in reported food allergies and fatal anaphylaxis. Although children usually become tolerant to most food allergens including soybean proteins, peanut allergy is rarely outgrown. The factors determining the allergenicity of molecules and therefore the wide variability among individuals are still unknown.

Numerous proteins from soybeans and peanuts have been shown to be allergenic to humans. Members of seed storage proteins identified as vicilins (7S) and glycinins (11S) in soybeans share considerable amino acid sequence homology to proteins in peanuts. For example, Ara h 1, a major peanut allergen, has been sequenced and shown to have considerable sequence homology to the 7S conglycinin family of soybean seed storage proteins. Ara h 3 has recently been shown to have sequence homology to the glycinins, members of the 11S nonglycosylated soybean family. As discussed below, we have also identified a glycinin subunit, A2B1a, using serum from soybean sensitive individuals for analysis of 2-D SDS-PAGE immunoblots.

The comparison and analytical analysis of allergens from soybean and peanut should provide information on the allergens with respect to allergenicity differences between these two legumes causing hypersensitivity responses. A comparison IgE-binding regions of proteins identified in peanut extracts as soybean homologs using soybean- and peanut-specific serum from sensitive individuals revealed significant differences in the amino acid sequences bound by the respective sera.

We have identified a seed maturation protein using soybean serum from sensitive individuals for screening a soybean seed cotyledon cDNA expression library. Five clones representing two 1500 and three 1400 bp fragments were isolated using this technology. Nucleotide sequence homology of clone 3a (1500 bp) and 4a (1400 bp) revealed them to have shared identity to a 51 kD maturation protein functioning as a desiccant protection protein in maturing soybean seeds. This, to our knowledge, is the first identification of this molecule as an IgE-binding protein. Hybridization experiments with oligonucleotides derived from peanut allergens used to detect allergens sequences in our cDNA library have been less promising.

Using 2-D SDS-PAGE/immunoblot analysis of enriched fractions from an SDS-PAGE prep gel procedure, we isolated several IgE-binding bands. A 20-22 kD band has been identified as the B1a fraction of the soybean glycinin subunit, A2B1a. This glycinin and several other IgE-binding proteins from this analytical procedure are being compared to homologous peanut allergens to determine the IgE-binding regions shared or unique to the respective legume hypersensitivity.

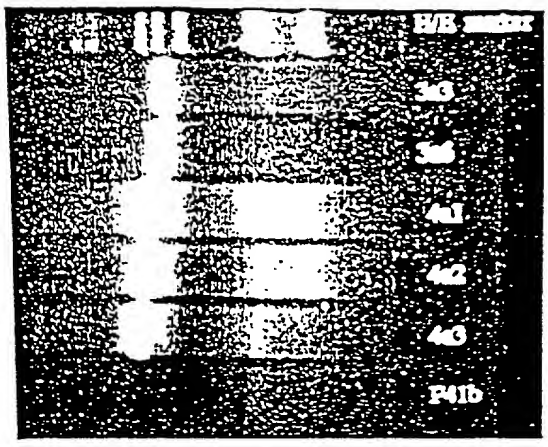
HOMOLOGOUS PROTEIN IDENTIFICATION

In the course of amino acid analysis of peanut allergens, several proteins were identified with sequence homology to glycinins and vicilins. One of the major peanut allergen, Ara h 1, was identified with an amino acid sequence homology (47% identity) to a soybean vicilin family of proteins, β-conglycinin. The α-chain was selected for linear epitope analysis using soybean and peanut-specific serum from sensitive individuals. A set of 15-mers offset by 8 amino acids were prepared using the SPOTS method for peptide synthesis. The membrane was blocked and incubated with a serum pool made up of soybean-sensitive individuals, washed, and incubated with radiolabeled anti-IgE, washed and exposed to X-ray film. Developed films were then assessed for IgE-positive binding regions. Following identification of soybean IgE positive binding regions, the SPOTS membrane was stripped according to manufacturer's instructions and re-probed with a serum pool made up of peanut-sensitive individuals. The respective IgE-binding regions were then identified as: Soybean with Peanut-IgE binding regions, Peanut with Soybean-IgE binding regions, Soybean, and Peanut-specific regions. For each region identified, 10-mers offset by 2 were synthesized and analyzed for specific IgE-binding amino acid sequences. See Figures 33 and 34.

cDNA CLONING

Soybean seeds, Glycinus max, Hutchinson variety, were obtained from a local health food store, frozen in liquid nitrogen, ground to a fine powder, and the RNA extracted using the method of Nedergaard et al (Mol Immunol 29:703,1992). Briefly, 2 g frozen seed powder was added to 10 mls buffer (250 mM sucrose, 200 mM Tris-HCl, pH 8.0, 200 mM KCl, 30 mM $MgCl_2$, 2% polyvinylpyrrokidone-40 and 5 mM 2-mercaptoethanol) and equilibrated with 10 ml fresh phenol (4°C). The suspension was homogenized and 10 ml of chloroform added with shaking for 5 min at RT. Phases were separated by centrifugation, 10k g for 20 min at 4°C and the aqueous phase transferred to a clean test tube and extracted 2x with equal volumes of chloroform/phenol. Nucleic acids were precipitated with sodium acetate/ethanol at -20°C overnight. The precipitates were collected by centrifugation at 13k g for 20 min at 4°C, washed with 70% ethanol and dried. Samples run in parallel were pooled in water and made 3M in LiCl, and the RNA precipitated for 4 hr at -20°C. The precipitate was collected by centrifugation outlined above and resuspended in distilled water. Fifty microliters of the RNA suspension was withdrawn for OD260/280 measurements and the RNA analyzed by agarose gel electrophoresis. Three aliquots representing a total of approximately 3.0 mgs total RNA was sent to STRATAGENE for purification of mRNA and the preparation of a Uni-Zap XR custom library.

The expression custom library was screened with serum from soybean-sensitive individuals and positive clones subcloned to homogeneity with respect to IgE-binding. Five clones were isolated from an initial screen and the plasmids purified from LB/ampicilin broth cultures using an Ameresco kit. The plasmid DNA from each clone was PCR amplified and analyzed in agarose gels. Two plasmid preparations had relative bp of approximately 1400 and the remaining three 1500 bp.



PCR AMPLIFIED PLASMIDS ISOLATED FROM SOYBEAN CDNA EXPRESSION LIBRARY

cDNA IgE + clones from seed cotyledons
Glycinus max, Hutchinson variety
In House RNA/STRATAGENE Library

Clone 4a:

Glycinus max Shi-Shi 51 kDa seed maturation protein: 96.5% identity in 114 bp overlap; begin at 423 end at 535.

Clone 3a:

Glycinus max Shi-Shi kDa seed maturation protein: 88.2% identify in 76 bp overlap; begin at 187 end at 262.

Glycinus max Shi-Shi amino acid sequence

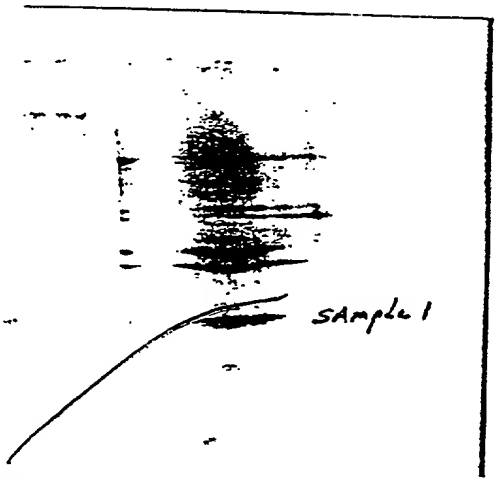
MASKVVSVLV IAMMLFAMNC NCTSVGHMPS TKEEGHDFQE SKAKTTQTAN
KAMETGKEGQ EAAESWTEWA KEKLSEGLGF KHDQESKEST TNKVSDYATD
TAQKSKDYAT DTAQKSKDYA GDAAQKSKDY AGDAAQKTKD YASDTAQTSK
DYAGDAAQKS KGYVGDAAQK TKEYVGDAAQ KTKDYATDAA QKTKDYATQK
TKDYASDATD AAKKTKDYAA QKTKDYASEA SDVAQNTKDY AAQKTKDYAS
GGAQKTKDYA SGGAQKTKDY ASDAAQKTKD YASDGAQKSK EYAGDVALNA
KDYAQKSKDY AGDAAQNVKD YASDAVQKRK EYSGDASHKS KEASDYASET
AKKTKDYVGD AAQRSKGAAE YASDAAQRKE YAGDATKRSK EASNDHANDM
AQKTKDYASD TAQRTKEKLQ DIASEAGQYS AEKAREMDAA AEKASDIAKA
AKQKSQEVKE KLGGQHRDAE L

Note: the same gene family with pGmPM8; a group III LEA protein with a putative signal peptide; 32 repeats of 11-mer with the consensus sequence KYDAGDAAQKT.
Function: desiccation protectant
Product: 51 kDa seed maturation protein.

ALLER EN IDENTIFICATION BY 2-I DS-PAGE

A crude soybean extract was applied to a 12.5% preparative SDS-PAGE gel and electrophoresed using a BIO-RAD prep cell. Five ml fractions were collected and aliquots were electrophoresed into a Pharmacia 24-well 10% horizontal gel, electrophoretically transferred to a nitrocellulose membrane, the remaining sites blocked using PBS/0.05% Tween 20, and analyzed for IgE-binding using serum from soybean-sensitive individuals. Fractions that bound IgE were dialyzed against 100mM ammonium bicarbonate (x4 x 4 liters) for 24 hours, lyophilized, reconstituted in distilled water and analyzed by 2-D (isoelectric focusing in the first dimension, pH 3-7, followed by a 4-20% SDS-PAGE gel molecular weight separation in the second) in duplicate. The proteins in the duplicate gels were transferred to nitrocellulose membranes, one was stained with Coomassie blue for protein identification and the other was prepared for IgE immunoblot analysis. IgE-binding proteins were identified by radiolabeled anti-IgE and X-ray autoradiography. Positive IgE-binding proteins by autoradiography were compared to the Coomassie stained gel protein profile. The stained blot was submitted to the Yale Biotechnology Center for amino acid sequencing. The results of this analysis revealed a 20-22kD protein with significant homology to the A2B1a glycinin protein family. Additional samples are being assessed for activity and identification.

A: Coomassie blue stained 2-D SDS-PAGE gel



B: IgE immunoblot of 2-D SDS-PAGE blot

SDS-PAGE identification of IgE + bands
Yale amino acid sequences

Sample 1: Mixed; primary (bottom) sequence matched various soybean glycinins.

							(Pro)	
							(Ser)	
							(Ala)	
(Gly)							(Asn)	
(His)			Leu			(Leu)	(Glu)	(Asn)
(Lys)	(Gly)		Asp			(Gly)	(Leu)	(Leu)
(Ser)	Ile	(Asp)	Glu	(Thr)	Ile	X	Thr	Met
1	2	3	4	5	6	7	8	9

			(His)					
			(Ile)					
			(Pro)					
			(Arg)					
			(Ser)					
			(Leu)					
			(Ala)					
(Lys)	(Arg)		(Asn)					
(Arg)	Leu	X	(Gln)	Asn	Ile	X	(Gln)	Thr
10	11	12	13	14	15	16	17	19

Sample 2:Primary (bottom) sequence matched soybean glycinin A2B1a and G2 precursors.

	(Val)	(Glu)		(Asn)				(Leu)
Gly	Ile	Asp	Glu	Thr	Ile	(Ala)	Thr	Met
1	2	3	4	5	6	7	8	9

		(Ala)						
	(Arg)	(Asn)	Ala			(Aka)		(Thr)
Arg	Leu	Arg	Gln	Asn	Ile	Gly	Gln	(Asn)
10	11	12	13	14	15	16	17	18

(Thr)								
(Pro)								
(Leu)		(Val)						
(Ala)		(Ala)						
(Asn)		(Gly)						
(Ser)	X	(Pro)						
19	20	21						

Sample 3: Primary (bottom) seqeunce matched glycinin A2B1a subunit precursor and G2 precursor from soybean.

	(Phe)							(Pro)
	(Leu)							(Asn)
	(Val)	(Glu)	(Leu)	(Asn)			(Gln)	(Leu)
Gly	Ile	Asp	Glu	Thr	Ile	(Ala)	Thr	Met

1	2	3	4	5	6	7	8	9
								(Asp) (Ala) (Thr) (Leu) (Pro) (Gln) Asn 18
(Pro) (Arg) 10	(Ala) (Arg) Leu 11	(Ala) (Arg) 12	(Arg) (Asn) (Asp) Gln 13	Asn 14	(Ser) Ile 15	(Ala) Gly 16	Gln 17	
Ser 19	(Gly) (Ala) (Ser) 20	X 21	(Pro) (Asn) Asp 22	(Phe) (Val) (Ala) (Asn) (Asp) Ile 23	Tyr 24	(Leu) (Ala) Asn 25		

GLYCININ PROTIEN A2B1A
REGION IN BOLD IDENTIFIED BY AMINO ACID SEQUENCE FROM 2-D SDS-PAGE

A2B1a AMINO ACID SEQUENCE:

MAKLVLSLCF LLFSGCFALR EQAQQNECQI QKLNALKPDN RIESEGGFIE TWNPNNKPFQ CAGVALSRCT LNRNALRRPS
YTNGPQEIYI QQGNGIFGMI FPGCPSTYQE PQESQQRGRS QRPQDRHQKV HRFREGDLIA VPGVAWWMYN NEDTPVVAVS
IIDTNSLENQ LDQMPRRFYL AGNQEQEFLK YQQQQQGGSQ SQKGKQQEEE ENEGSNILSG FAPEFLKEAF GVNMQIVRNL
QGENEEEDSG AIVTVKGGLR VTAPAMRKPQ QEEDDDDEEE QPQCVETDKG CQRQSKRSRN **GIDETICTMR LRQ**NIGQNSS
PDIYNPQAGS ITTATSLDFP ALWLLKLSAQ YGSLRKNAMF VPHYTLNANS IIYALNGRAL VQVVNCNGER VFDGELQEGG
VLIVPQNFAV AAKSQSDNFE YVSFKTNDRP SIGNLAGANS LLNALPEEVI QHTFNLKSQQ ARQVKNNNPF SFLVPPQESQ
RAVA

22 kD soybean SPOT/Ara h 3 amino acid sequence comparison:

```
GIDETICTMR LRQNIGQNSS PDIYNPQAGS ITTATSLDFP ALWLLKLSAQ YGSLRKNAMF
GIEETICTAS ALLNIGRNRS PDIYNPQAGS LKTANDLNLL ILRWLGLSAE YGNLYRNALF
** *****      *** * * **********   **  *        * ***  ** *  ** *


VPHYTLNANS IIYALNGRAL VQVVNCNGER VFDGELQEGG VLIVPQNFAV AAKSQSDNFE
VAHYNTNAHS IIYRLRGRAH VQVVDSNGNR VYDEELQEFJ VLVVPQNFAV AGKSQSENFE
* **  ** * *** * ***  ****  ** * * * ****   ** ******* * **** ***


YVSFKTNDRP SIGNLAGANS LLNALPEEVI QHTFNLKSQQ ARQVKNNNPF SFLVPPQESQ
YVAFKTDSRP SIANLAGENS VIDNLPEEVV ANSYGLQREQ ARQLKNNNPF KFFVPPSQQS
** ***  ** ** **** **     *****       *   * *** ******  * ***

RRAVA
PRAVA
 ****
```

PROTEIN A2B1A
IDENDITIFIED BY AMINO ACID SEQUENCE FROM 2-D SDS-PAGE
15-MER X 8 IgE POSITIVE REGIONS

Region 1 2

MAKLVLSLCF LLFSGCFALR EQAQQNECQI QKLNALKPDN RIESEGGFIE TWNPNNKPFQ

CAGVALSRCT LNRNALRRPS YTNGPQEIYI QQGNGIFGMI FPGCPSTYQE PQESQQRGRS

3

QRPQDRHQKV HRFREGDLIA VPGVAWWMYN NEDTPVVAVS IIDTNSLENQ LDQMPRRFYL

AGNQEQEFLK YQQQQQGGSQ SQKGKQQEEE ENEGSNILSG FAPEFLKEAF GVNMQIVRNL

4

QGENEEDSG AIVTVKGGLR VTAPAMRKPQ QEEDDDDEEE QPQCVETDKG CQRQSKRSRN

5

GIDETICTMR LRQNIGQNSS PDIYNPQAGS ITTATSLDFP ALWLLKLSAQ YGSLRKNAMF

VPHYTLNANS IIYALNGRAL VQVVNCNGER VFDGELQEGG VLIVPQNFAV AAKSQSDNFE

6

YVSFKTNDRP SIGNLAGANS LLNALPEEVI QHTFNLKSQQ ARQVKNNNPF SFLVPPQESQ

RRAVA

Soybean IgE positive binding regions

N-terminal amino acid sequence from 2-D SDS-PAGE

A2B1A REGIONS
10-MERS X 2 IgE-POSITIVE

Region 1:
MAKLVLSLCF LLFSGCFALR EQA

Region 2:
KPFQ CAGVALSRCT LNRNALRRPS YTNGPQEIYI QQGNGIFGMI FPGCPSTYQE P

Region 3:
NQ LDQMPRRFYL AGNQEQEFLK YQQQQQGGSQ SQKGKQQEEE ENEGS

Region 4:
SG AIVTVKGGLR VTAPAMRKPQ Q

Region 5:
GS ITTATSLDFP ALWLLKLSAQ YGSLRKNAMF VPHYTLNANS IIYALNGRAL VQV

Region 6:
VI QHTFNLKSQQ ARQVKNNNPF S

CONCLUSIONS

PEANUT AND SOYBEAN ALLERGENS HAVE UNIQUE AS WELL AS SHARED AMINO ACID SEQUENCES IN THE VICILIN AND GLYCININ SOYBEAN FAMILY OF SEED STORAGE PROTEINS

THE EXPRESSION cDNA SOYBEAN SEED COTYLEDON LIBRARY CAN BE USED TO IDENTIFY IgE-BINDING PROTEINS

Figure 37

IgE BINDING OF rAra h 2 PROTEINS IN WESTERN BLOT ANALYSIS

Equal amounts of purified wild type and mutated Ara h 2 proteins were separated by gradient (4-20%) PAGE and electrophoretically transferred onto nitrocellulose paper. The blots were incubated with antibody directed against N-terminal T7-tag or serum from peanut sensitive patients.

Panel A, Western blots of WT (10 wild type epitopes), MUT4 (6 wild type epitopes), and MUT10 (0 wild type epitopes) incubated with T7 tag antibody or patient serum

Panel B, IgE binding of mutated rAra h 2 proteins compared to the WT in Western blot analysis by individual patient sera. Laser densitometer was used to quantitate relative IgE binding. Each line represents IgE binding for the individual patient.

The Effect of Digestion on the Allergenicity of the Major Peanut Allergen Ara h 1. RA. Kopper, S. Maleki, HA Sampson, AW. Burks, and GA. Bannon. Dept. of Pediatrics and Biochemistry & Molecular Biology, University of Arkansas for Medical Sciences, Little Rock, AR 72205; Dept. of Pediatrics, Mt. Sinai Medical School, New York, NY.

Peanut allergy is a chronic, potentially severe reaction for which there is currently no treatment other than strict avoidance of peanut and all peanut products. Hypersensitivity to peanuts is an IgE-mediated response to one or more of three major and several minor peanut protein allergens. These proteins appear to be especially resistant to hydrolysis by digestive enzymes. Among these allergenic proteins, Ara h 1 is a major contributor to this immunologic disorder because of its relative abundance and allergenicity. This laboratory has previously identified the Ara h 1 protein and characterized its occurrence as a stable homotrimer. The stability of Ara h 1, the role of its glycosylation, and the effect on its IgE-binding capacity as a result of proteolytic digestion have now been investigated. Ara h 1 is stable at pH ranges that would be encountered in the digestive tract (pH 2-9). Yet, even in its native trimeric form, it is hydrolyzed by proteolytic digestive enzymes. However, many of the potential specific cleavage sites in the Ara h 1 molecule are protected from these proteases. Several of the peptide fragments produced upon treatment of Ara h 1 with pepsin, trypsin, and chymotrypsin are very resistant to further proteolytic digestion. Many of these stable peptide fragments bind significant amounts of IgE. These results were confirmed using actual human gastric fluid. Those fragments that survive digestion and carry important IgE-binding epitopes may play a major role in the allergic response to peanuts. Experiments are currently underway to determine the sequence of the surviving peptides and how the known IgE-binding epitopes map within these fragments.

Introduction

Legume seed storage proteins constitute the third largest source of dietary protein on earth. They are of particular importance as a nutritional source in developing countries that lack ample supplies of animal proteins. Peanuts are widely used for the preparation of a variety of foods in the United States and are also relied on as a protein extender in developing countries. There has been an increase in the observed incidence of peanut allergies in children over the last 10 years, possibly due to the increased use of peanut products in infant diets. While children become tolerant to most other food allergies with age, peanut allergy is rarely outgrown. Thus, it is increasingly common for the public to be exposed to an abundantly utilized and often disguised food such as peanuts. This has led to increasing rates of sensitization, accidental ingestion, anaphylaxis and even death in peanut allergic patients.

The general biochemical characteristics of most food allergens indicate that they are low molecular weight glycoproteins (<70kDa) with acidic isoelectric points that are highly abundant in the food. These proteins are usually resistant to proteases, heat, and denaturants allowing them to resist degradation during food preparation and digestion. There are a number of characteristics that increases a food allergen's capacity to provoke a dangerous systemic allergic reaction. These include its ability to stimulate high titers of IgE and to resist degradation sufficiently to produce fragments containing multiple IgE binding epitopes. A high titer of IgE ensures that mast cells will accumulate enough allergen specific IgE to trigger degranulation when cross-linked. Likewise, allergen fragments containing multiple IgE binding epitopes are able to cross-link and activate multiple FcεR1 receptors. An allergen fragment must have at least two IgE binding epitopes in order to cross-link and activate multiple FcεR1 receptors. The more degraded an allergen becomes

the more fragments are produced that contain single IgE binding epitopes. Thus, the biochemical and structural aspects of allergens play a critical role in the disease process.

The peanut allergen Ara h 1 is a vicilin-like seed storage protein found in the cotyledon. This protein is one of the main storage proteins of the seed which is utilized as a nitrogen and amino acid source during development of a new peanut plant. In addition to its importance to the developing plant, Ara h 1 is recognized by serum IgE from >90% of peanut-sensitive patients, thus establishing it as an important allergen in the etiology of this disease. The linear IgE-binding epitopes of this allergen have been mapped and shown to consist of 23 binding sites. These sites are evenly distributed along the linear sequence of the molecule. However, a molecular model of the tertiary structure of the middle and C-terminal domains of the Ara h 1 protein shows that the IgE-binding sites were clustered into two main regions. In addition, Ara h 1 forms homo-trimers, a physical characteristic that may be important in establishing it as an allergen.

Figure 38 Model of Ara h 1 Tertiary Structure

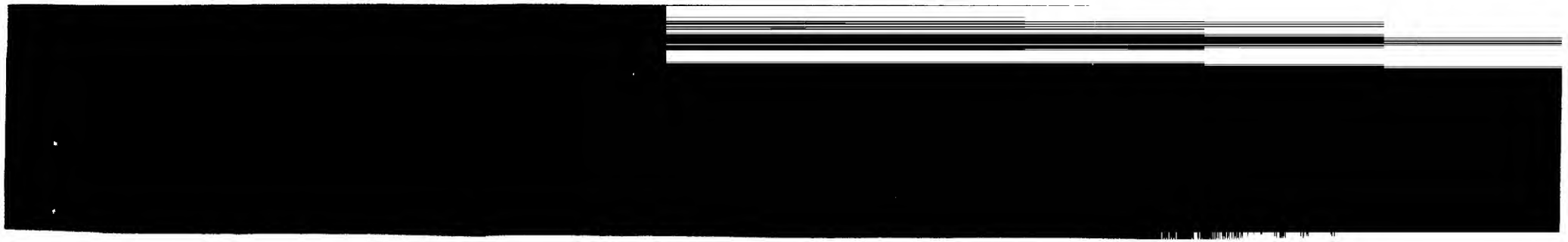
Upper Panel: A space filled model of the middle and C-terminal domains of the Ara h 1 allergen is shown. The red areas represent the IgE binding epitopes. The yellow atoms represent residues that were determined to be critical for IgE binding to occur. The numbers correspond to some of the epitopes for ease of orientation.

Lower Panel: A space-filled model of the Ara h 1 trimer is shown. The red areas represent the IgE binding epitopes. Oligomerization brings the two-clustered epitope regions seen in the monomeric model together. Formation of the trimer buries some of the epitopes that were exposed in the monomer suggesting that dissolution of the trimer is required for recognition of some of the epitopes by the immune system.

Figure 39 Ara h 1 Amino Acid Sequence Showing IgE Binding Epitopes and Protease Digestion Sites

Upper Panel: The primary amino acid sequence of the Ara h 1 protein is shown as the one letter amino acid code. The red amino acids indicate the recognition sites for pepsin and chymotrypsin cleavage. The boxed sequences are the IgE binding epitopes.

Lower Panel: The primary amino acid sequence of the Ara h 1 protein is shown as the one letter amino acid code. The red amino acids indicate the recognition sites for trypsin cleavage. The boxed sequences are the IgE binding epitopes.



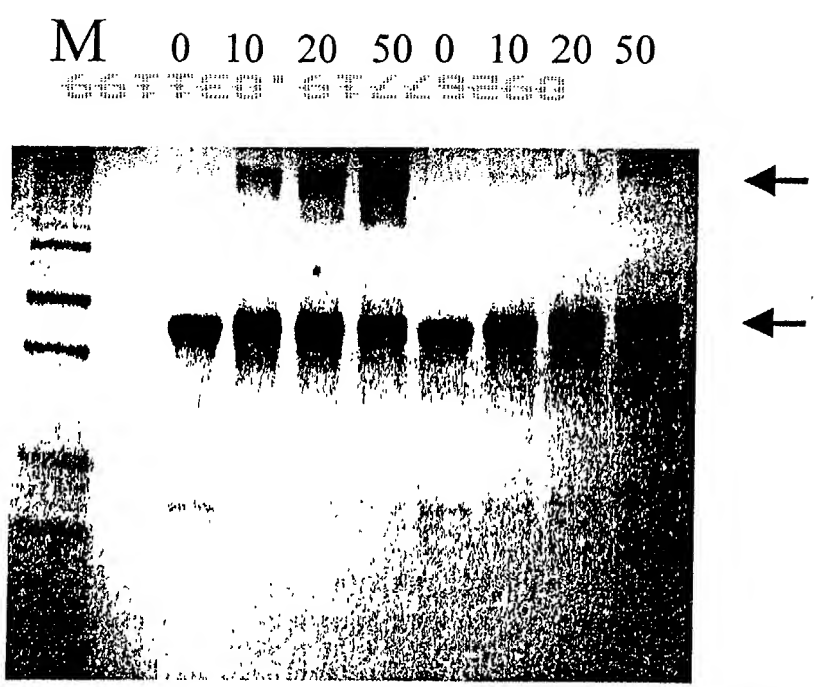


Figure 40 The Ara h 1 trimer is unstable at pH2.

In order to assess the stability of the Ara h 1 trimer at pHs that would be encountered in the human stomach, cross-linking experiments were performed using purified Ara h 1 protein suspended in a pH 2.1 buffer. Purified Ara h 1 (2 mM) was suspended in 500 µl of either a pH 2.1 buffer or a pH 7.6 buffer and allowed to incubate for one hour at room temperature. Cross-linking was performed using 5% DSP in DMF for varying lengths of time (10, 20, or 50 seconds). Results indicate that the Ara h 1 trimer is unstable at acidic pHs that would be encountered in the human stomach but that the monomer is stable at this pH. Further experiments indicate that the monomer is stable at pH 2.1 for greater than 8 hours at 37°C.

Peanut allergy (hypersensitivity) is an adverse immunological reaction following the ingestion of peanut-containing foods. It is a serious health problem because of the severity of the reaction, lack of immunotherapy, and difficulties in avoiding peanut containing products. Sensitized individuals produce IgE, which can bind to antigenic determinants (epitopes) on peanut-specific allergens. A major peanut allergen Ara h 2 is recognized by serum IgE from more than 90% of peanut-sensitive patients. To study the relationship between Ara h 2 primary structure and its allergenicity we produced a recombinant Ara h 2 protein (rAra h 2) *in E. Coli.* The recombinant protein was recognized by serum IgE from all peanut allergic patients tested in Western blot analysis. Linear IgE binding epitopes of Ara h 2 as well as amino acids in these epitopes, which were critical for IgE binding, were identified using solid-phase peptide synthesis. To modulate IgE reactivity of the allergen we constructed rAra h 2 protein with mutations in the immunodominant IgE binding epitopes. The abilities of the wild type and mutant rAra h 2 proteins to react with IgE have been tested in Western blot analysis with sera from 20 peanut sensitive individuals. IgE reactivity of mutant rAra h 2 was significantly decreased compared to the wild type. The allergens also were used in T-cell proliferation assays. Both wild type and mutant proteins retained the ability to stimulate T-cell proliferation at comparable levels. We hypothesize that modified allergens, which have decreased allergenicity, but retain their ability to stimulate an immune response, can be used as a safe immunotherapeutic for the treatment of peanut allergic individuals.

INTRODUCTION

Adverse reactions to foods may affect up to 8% of children less then 3 years of age and 1% to 2% of the general population. The major sources of food allergens are eggs, milk, peanuts, and soy, which are responsible for about 80% of the hypersensitivity reactions caused by foods. The severity of reactions to peanuts and difficulties in avoidance of peanut-containing products necessitates development of new methods of prevention, diagnosis and treatment of peanut hypersensitivity. The mechanism of food hypersensitivity involves IgE mediated reactions. IgE production is affected by genetic predisposition, by the history of exposure to the allergen, and by the nature the allergen itself. It is not known at the present time why some antigens elicit strong allergenic response, whereas others are not allergenic. Identification and characterization of allergens is important for understanding their role in development of food hypersensitivity.

Ara h 2, a major peanut allergen, is recognized by serum IgE from more then 95% of peanut-sensitive patients. In previous work we identified 10 linear IgE binding epitopes on this protein, and determined amino acids critical for IgE binding in each of these epitopes. This report is focused on modulation of the allergenicity of the Ara h 2 protein by site-directed mutagenesis of its IgE binding epitopes.

Table 6

Ara h 2 IgE BINDING EPITOPES AND AMINO ACIDS CRITICAL FOR IgE BINDING

PEPTIDE	AA SEQUENCE	*Ara h 2* POSITION
1	HASARQQWEL	15-24
2	QWELQGDRRC	21-30
3	DRRCQSQLER	27-36
4	LRPCEQHLMQ	39-48
5	KIQRDEDSYE	49-58
6	YERDPYSPSQ	57-66
7	SQDPYSPSPY	65-74
8	DRLQGRQQEQ	115-124
9	KRELRNLPQQ	127-136
10	QRCDLDVESG	143-152

The Ara h 2 IgE binding core epitopes (1-10) are underlined. The positions of each peptide with respect to the *Ara h* 2 protein are indicated in the right hand column. Amino acids important for IgE binding are shown in bold letters.

Table 7

RECOMBINANT Ara h 2 PROTEINS

T7 tag

MASMTGGQMG RDPNSARQQ**W** ELQGDRRCQS
QL**E**RANLR**P**C EQHLMQKIQR **D**EDSYER**D**PY
SPSQ**D**PYSPS PYDRRGAGSS QHQERCCNEL
NEFENNQRCM CEALQQIMEN QSDRLQG**R**QQ
EQQFKRE**L**RN LPQQCGLRAP QRCD**L**DVESG
GRDRYAAALE HHHHHH

His tag

Amino acids important for IgE binding were mutated to alanine by single-stranded mutagenesis (epitopes 3, 4, and 6) or by PCR (epitopes 1,2,5,7,8,9,10). Mutations were confirmed by sequence analyses of Ara h 2 cDNA clones.

Amino acids replaced by alanine in MUT4 are shown in bold and underlined letters.

Amino acids replaced by alanine in MUT10 are shown in bold letters.

Figure 41
EXPRESSION AND PURIFICATION OF RECOMBINANT Ara h 2 PROTEINS

The cloned wild type or mutated Ara h 2 genes were used to produce recombinant Ara h 2 proteins. The portion of Ara h 2 sequence excluding the first 54 nucleotides, which encode the signal peptide, was amplified by PCR. The PCR product was ligated to the EcoRI-NotI sites of pET 24 (a). This vector encodes a T7-tag at the N-terminus and His-tag at the C-terminus of expressed fusion proteins. *E.coli* BL21(DE3) cells were transformed with the Ara h 2 constructs and exponentially growing cells were induced with 1 mM IPTG. Cells were pelleted and the recombinant Ara h 2 proteins were purified by affinity chromatography on a nickel-resin column.

Panel A, Schematic representation of recombinant Ara h 2 protein expression in *E.coli*

Panel B, SDS-PAGE of fractions, obtained during purification of recombinant Ara h 2 proteins on the Ni-column: lane 1-cell lysate, lane 2-unbound fraction, lane 3-20mM imidazole wash fraction, lanes 4-6- 100mM imidazole elution fractions.

Figure 37
IgE BINDING OF rAra h 2 PROTEINS IN WESTERN BLOT ANALYSIS

Equal amounts of purified wild type and mutated Ara h 2 proteins were separated by gradient (4-20%) PAGE and electrophoretically transferred onto nitrocellulose paper. The blots were incubated with antibody directed against N-terminal T7-tag or serum from peanut sensitive patients.

Panel A, Western blots of WT (10 wild type epitopes), MUT4 (6 wild type epitopes), and MUT10 (0 wild type epitopes) incubated with T7 tag antibody or patient serum

Panel B, IgE binding of mutated rAra h 2 proteins compared to the WT in Western blot analysis by individual patient sera. Laser densitometer was used to quantitate relative IgE binding. Each line represents IgE binding for the individual patient.

CONCLUSIONS

- **Allergenicity of a recombinant Ara h 2 protein, expressed and purified from *E. coli* is comparable to that of the native protein, as shown by IgE binding in Western blot and inhibition analyses and T-cell proliferation assays**

- **Linear epitopes play a major role in IgE binding of a peanut allergen, Ara h 2, and single amino acid mutations in each epitope may result in loss of IgE binding.**

- **T-cell epitopes may be affected by mutagenesis of overlapping IgE binding epitopes**

PERSPECTIVES

- **Improved diagnostics using recombinant allergens**
- **Immunotherapy with modified, "hypoallergenic" forms of allergens**
- **Development of transgenic crops with decreased allergenicity**

Modulation of the reactivity of the major peanut allergen Ara h 1through epitope characterization, structural analysis, and mutation. DS. Shin, CM compadre, HA. Sampson, AW. Burks, and GA. Bannon. Department of Biochemistry & Molecular Biology, The Biomedical Visualization Center, Department of Pediatrics, University of Arkansas for Medical Sciences, Little Rock, AR, 72205; Department of Pediatrics, Mt. Sinai School of Medicine, New York, NY 10069.

Allergy to peanuts is a significant health problem because of high prevalence, lack of treatment and potentially fatal reactions. Twenty-three B-cellepitopes and the critical amino acids involved inIgE binding to the major peanut allergen Ara h 1 have been identified. Molecular modeling studies of the conserved middle and C-terminal domains of the Ara h 1 monomer revealed that the majority of the linear epitopes within these domains (epitopes 10-22) were clustered near the regions of the two terminal alpha-helical bundles and therefore may represent two conformational epitopes. It was determined that Ara h 1, like other vicilin proteins, forms a trimeric structure. Further modeling studies showed that the two clustered regions overlap and thus some of theIgE-binding regions are buried suggesting that the protein is recognized by the immune system in both native and denatured forms. We are currently attempting to construct a non-IgE reactive recombinant Ara h 1 clone. Therefore, computational modeling studies were performed to determine which critical amino acids are less important for stability of the protein and less likely to interrupttrimer formation. It was found that less conserved residues were optimal candidates for mutagenesis. We constructed a mutant recombinant protein with single alanine point mutations in epitopes 1,2,3,4,5,6, and 17. Epitopes 1-6 were chosen because they lie within the variable N-terminal domain and are not conserved between the vicilins and therefore may be responsible for the peanut's extreme allergenicity. Assays utilizing serum from patients with peanut hypersensitivity and thewild-type and mutant recombinant proteins revealed a significant decrease inIgE binding to the mutant protein in 50% of the patients tested. Future studies involve furthermutagenesis of candidate residues to develop a hypoallergenic vicilin protein that may be utilized in immunotherapeutic regimens for peanut allergic individuals.

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Introduction

It is estimated that 5 to 8% of children below the age of three have food allergies. Roughly, 90% of serious allergic reactions occur in response to peanuts, soybeans, crustacea, fish, cow's milk, eggs, treenuts and wheat. Unlike most food allergies that spontaneously resolve, those to peanut usuallypersists for a lifetime and account for the majority of near-fatal and fatal reactions. The current therapeutic method to desensitize hypersensitive individuals to inhaled allergens is through weekly or biweekly subcutaneous injection of extracts derived from the allergen source. It is believed that the allergens within theextracts tolerize T-cells and thus lead T-cells to switch their secreted cytokine profile. In the case of food allergies, the use of extract injection is generally avoided as a treatment because of the increased risk of severe anaphylaxis. Therefore, the only recommended therapy for food allergy is avoidance (*T*). Because recognition of allergen polypeptide sequences by both T-cells and B-cells plays such a crucial role in the mechanisms of the disease, polypeptides bearing T-cellepitopes or recombinant allergens with modified B-cell epitopes have been targeted as immunotherapy candidates.

Ara h 1, a vicilin family seed storage protein, represents a major peanut allergen. Twenty-three B-cell epitopes within Ara h 1 have been mapped through synthetic peptide-IgE binding assays. Each individual patient recognizes a different number and combination of epitopes with varying degrees of binding affinity. Fourepitopes, designated 1, 3, 4 and 17, are immunodominant in terms of recognition where >80% of tested individuals haveIgE which recognizes these epitopes. The amino acids which are critical for IgE recognition within 21 of these epitopes have been identified. These residues when substituted individually with another amino acid either nearly or completely abolish IgE binding to synthetic peptides representing theepitopes. There is an average of almost 3 residues per epitope that have this property.

We are attempting to create a recombinant Ara h 1 protein that has point mutations within the B-cellepitopes which would lead to loss of IgE binding. Such a protein could be used safely in standard immunotherapy or, alternatively, can be reintroduced into the plant to decrease the reactivity of the peanut itself. In order to accomplish our goal, this study focused on producing a pilot mutagenized recombinant protein, with several substitutions including residues within the immunodominant epitopes, and testing the reactivity of the clone in both *in vitro* assays.

Peptide	Amino Acid Sequence	Position
1	AKSSPYQKKT	25-34
2	QEPDDLKQKA	48-57
3	LEYDPRLVYD	65-74
4	GERTRGRQPG	89-98
5	PGDYDDDRRQ	97-106
6	PRREEGGRWG	107-116
7	REREEDWRQP	123-132
8	EDWRRPSHQQ	134-143
9	QPRKIRPEGR	143-152
10	TPGQFEDFFP	294-303
11	SYLQGFSRNT	311-320
12	FNAEFNEIRR	325-334
13	EQEERGQRRW	344-353
14	DITNPINLRE	393-402
15	NNFGKLFEVK	409-418
17	RRYTARLKEG	498-507
18	ELHLLGFGIN	525-534
19	HRIFLAGDKD	539-548
20	IDQIEKQAKD	551-560
21	KDLAFPGSGE	559-568
22	KESHFVSARP	578-587

16

Table 8 Amino acids critical to IgE binding.

The Ara h 1 IgE binding epitopes are indicated as the single letter amino acid code. The position of each peptide with respect to the Ara h 1 protein coding sequence is indicated in the right hand column. The amino acids that, when altered, lead to loss of IgE binding are shown as bold, underlined residues. Epitopes 16 and 23 were not included in this study because they were recognized by single patients who were no longer available to the study.

Patient	Mutated Epitopes	Wild type epitopes	Relative Binding
1	1,4,5,17	8,13	DECREASED
2	2,3,4,17	14,18	EQUAL
3	4,5,17	11,14,18,19,20,22	INCREASED
4	2,4,5,17	9,23	DECREASED
5	1,4,17	9,10,12,13,14,15, 18,21,22	EQUAL
6	4,17	8,9,20,23	DECREASED
7	1,2,4,17	13	EQUAL
8	1,3,4,17	13,22	EQUAL
9	1,2,4,17	10	DECREASED
10	3,17	8,9,10,11	DECREASED

Table 7 Mutation of the Ara h 1 protein leads to decreased

IgE binding in 50% of cases tested.

Western blots of wild type and mutant recombinant proteins probed with individual peanut-sensitive patient s were performed. Data for each patient is numbered 1-10 in the first column. The second column lists theepitopes that each patient recognized that were changed in the mutant protein. The third column lists theepitopes that each patient recognized that were not changed in the mutant protein. The fourth column shows the relativeIgE binding affinity of the mutant protein vs. the wild type protein. In 50% of the casesIgE binding to the mutant protein was significantly decreased.

Conclusions

Amino acids critical to IgE binding have been identified in each of the linearepitopes of the major peanut allergen Ara h 1.

Serum IgE binding to Ara h 1 can be substantially reduced when 7/23 linearepitopes have been modified by single amino acid alterations.

Through the use of molecular models of tertiary structure amino acid alterations toAra h 1 can be chosen so as to minimize disruption of tertiary structure.

Perspectives

The work presented in this poster indicate that it is possible to produce a recombinantAra h 1 protein that will bind substantially lower amounts of serum IgE from peanut sensitive patients. This may represent a safe alternative therapeutic reagent that could be used to desensitize peanut allergic patients. Furthermore, this work shows that tertiary structure of the Ara h 1 protein will be an important consideration if a modified Ara h 1 gene is re-introduced to the peanut plant.

Funding provided by NIH and the Clarissa Sosin Research Foundation

Mutational Analysis of the IgE-Binding Epitopes of the Peanut Allergen, Ara h 3: a Member of the Glycinin Family of Seed-Storage Proteins. [1]P. Rabjohn, [2]AW. Burks, [3]HA. Sampson, and [1]GA. Bannon. [1]Dept. of Biochemistry and Molecular Biology and [2]Pediatrics, University of Arkansas for Biomedical Sciences, Little Rock, AR 72205; [3]Dept. of Pediatrics, Mt.Sinai Medical School, New York, NY 10029

Peanut allergy is a significant IgE-mediated disease affecting both children and adults. We have previously reported the cloning of the cDNA encoding a third peanut allergen, Ara h 3, with significant homology (70-80%) to the glycinin family of seed-storage proteins. The recombinant form of this protein was expressed in a bacterial system and was recognized by serum IgE from ~ 45% (8/18) of our peanut-allergic patient population. Serum IgE from these patients and overlapping, synthetic peptides were used to map the linear, IgE-binding epitopes of Ara h 3. Four epitopes, between 10-15 amino acids in length, were found within the primary sequence, with no obvious sequence motif shared by the peptides. One epitope appears to be immunodominant within the Ara h 3 population, in that it is recognized by all Ara h 3 – allergic patients. Mutational analysis of the epitopes revealed that single amino acid changes within these peptides could lead to a reduction or complete loss of IgE-binding. The location of the epitopes within the primary sequence of the protein is favorable for mutagenesis. None of the epitopes reside within the basic subunit, shown previously in soybean to be important for oligomer assembly, a requisite step in the ability of glycinins to function as seed-storage proteins. Also, all 26 invariant amino acids, thought to be important for the structural conservation of glycinins, lie outside of the IgE-binding epitopes. The elucidation of the major IgE-binding epitopes on Ara h 3 and the determination of which amino acids within these epitopes are critical for IgE-binding provides the information necessary to alter the Ara h 3 cDNA to encode a non-allergenic glycinin that retains its ability to function as a seed-storage protein.

INTRODUCTION

Peanut allergy is a major health concern due to the severity of the allergic reaction, the persistence of the allergic response throughout the lifetime of the individual, and the ubiquitous use of peanut as a protein supplement in processed foods. Approximately 1-3% of the USA population suffers from some form of food allergy. Peanuts, tree nuts, and shellfish are responsible for the majority of food hypersensitivity reactions in adults, while peanuts, milk, and eggs account for the majority of reactions in children. The reaction to peanut is generally more severe than the reaction to other foods, often resulting in fatal anaphylaxis. While most children outgrow allergies to milk and eggs, peanut allergies persist into adulthood, lasting the entire lifetime of the individual. Currently, avoidance is the only treatment for patients with peanut allergies. Unfortunately, the inclusion of peanut as a protein supplement in processed foods makes accidental consumption almost inevitable. Despite the prevalence of peanut hypersensitivity in children and an increasing number of deaths each year from peanut-induced anaphylaxis, the identification and characterization of unique, clinically-relevant allergens from peanut is incomplete, limiting our understanding of their role in the immunobiology of hypersensitivity reactions.

We have previously reported the cloning of the cDNA encoding a third peanut allergen, Ara h 3. The recombinant form of this protein has been expressed in a bacterial system and is recognized by serum IgE from 44% (8/18) of our peanut hypersensitive patient population. The linear, IgE-binding epitopes and the critical residues for IgE binding were identified by probing synthetic peptides representing the primary sequence of the allergen with serum from Ara h 3 – allergic individuals.

The goal of this research is to create a gene that can be used for both plant transformation and immunotherapeutic purposes. The elucidation of the major IgE-binding epitopes on Ara h 3 and the determination of which amino acids within these epitopes are critical for IgE-binding provides the information necessary to alter the Ara h 3 gene by site-directed mutagenesis to encode a protein that escapes IgE recognition.

Figure 50 **Immunoblot of purified recombinant Ara h 3 with serum IgE from individual patients.** The cDNA encoding Ara h 3 was expressed under control of the T7 *lac* promoter in a bacterial system. Lanes A-R represent purified recombinant Ara h 3 (~ 60 kD) probed with serum IgE from individual patients with documented peanut hypersensitivity. Approximately 45% (8/18) of the patients recognized the recombinant form of the Ara h 3 allergen. Lane S represents recombinant protein probed with a pool of serum IgE from peanut-allergic patients, while lane T represents recombinant protein probed with serum from a non-peanut-allergic individual with elevated IgE levels.

Figure 51 **Multiple IgE-binding regions identified on the Ara h 3 allergen.** *A*) The Ara h 3 primary sequence was synthesized as 15 amino acid-long peptides offset from each other by eight residues. These peptides were probed with a pool of serum IgE from peanut-hypersensitive patients. The position of the peptides within the Ara h 3 protein are indicated on the left-hand side of *A*. (+) indicates an immunodominant peptide from Ara h 2 that served as a positive control, while (-) indicates a peptide synthesized to serve as a negative control. *B*) The amino acid sequence of the Ara h 3 protein is shown. The shaded areas (R1-R4) correspond to the IgE-binding regions shown in the upper panel.

Figure 52 **Identification of a core IgE-binding epitope on the Ara h 3 allergen.** Detailed epitope mapping was performed on IgE-binding regions identified in Figure 51 by synthesizing overlapping peptides fifteen amino acids in length offset from the previous peptide by two residues. These peptides were probed with a pool of serum IgE from 8 patients previously shown to recognize recombinant Ara h 3. The data shown represents amino acids 299-321. (B) The amino acid sequence (residues 299-321) of Ara h 3 that was tested in *A* is shown. Residues in ~~boldface~~ correspond to common IgE-binding amino acids of the spots shown in *A*. shaded area

TABLE 10

Ara h 3 IgE-Binding Epitopes[a]

Epitope	AA Sequence	Ara h 3 Position	% recognition
1	IETWNPNNQEFECAG	33-47	25%
2	GNIFSGFTPEFLEQA	240-254	38%
3	VTVRGGLRILSPDRK	279-293	100%
4	DEDEYEYDEEDRRRG	303-317	38%

[a]The Ara h 3 IgE-binding epitopes are indicated as the single letter amino acid code. The position of each peptide with respect to the Ara h 3 protein coding sequence is indicated. The percent recognition is the percentage of patients previously shown to recognize recombinant Ara h 3 whose serum IgE recognized that particular synthetic epitope.

Figure 53 **Ara h 3 epitopes can be mutated to non-IgE-binding peptides.** Epitope 4 was synthesized with an alanine residue substituted for one of the amino acids at each position in the peptide. The synthesized peptides were probed with a pool of serum IgE from peanut hypersensitive patients whose IgE has previously been shown to recognize this peptide. The letters across the top of each panel indicate the one-letter amino acid code for the residue normally at that position and the amino acid substituted for that residue. The numbers indicate the position of each residue in the Ara h 3 protein. *WT* indicates the wild-type peptide with no amino acid substitutions.

Table II

Amino Acids Critical to IgE-Binding[a]

Epitope	AA Sequence	Ara h 3 Position
1	IETWN**PN**NQEFECAG	33-47
2	GNI**F**SG**F**TPE**FL**EQA	240-254
3	VTVRGG**L**R**IL**S**P**DRK	279-293
4	DEDEY**EYDE**E**DR**RRG	303-317

[a]The Ara h 3 IgE-binding epitopes are indicated as the single letter amino acid code. The position of each peptide with respect to the Ara h 3 primary sequence is indicated in the right-hand column. The amino acids that, when altered, led to a decrease in IgE-binding are shown as the bold, underlined residues.

Figure 54 Recombinant expression and Western blot analysis of the Ara h 3 mutant

A) The Ara h 3 cDNA was mutated by PCR to encode alanine for one critical residue within each epitope. The cDNA encoding the 40 kD acidic chain of the 11S legumin-like storage protein was placed under the control of the T7 lac promoter and expressed in a bacterial system. The WT recombinant Ara h 3 protein refers to the 60 kD preproglobulin consisting of covalently attached 40 kD (acidic) and 20 kD (basic) proteins. Both the mutated and wild-type recombinant proteins were purified by Ni^{2+} column chromatography. B) The proteins represented in (A) were blotted to nitrocellulose and probed with serum IgE from three patients previously shown to recognize recombinant Ara h 3. As seen from the blot, the mutated Ara h 3 40 kD protein was not recognized by serum IgE from the Ara h 3 – allergic patients.

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Various minor allergens and three major peanut allergens have been identified among which Ara h 2 is one of the most important. We describe a method for rapid purification of Ara h 2, which is recognized by a pool of serum IgE from peanut allergic patients, can cause T-cell proliferation and induce histamine release from the blood of these individuals. The peanut specific T-cell lines were all shown to be primarily CD4+ and secrete IL-4. Once Ara h 2 was shown to induce the proliferation of T-cells from allergic individuals, synthetic overlapping peptides of 20 amino acids long offset by 5 amino acids were made and used in epitope mapping studies. Five immunodominant T-cell epitopes were identified of which only one has a significant overlap with a previously identified major B-cell epitope. T cells from non-allergic individuals were also shown to recognize a subset of these epitopes but secreted lower levels of IL-4. We have also determined that crude peanut extract is able to activate a signal transduction cascade through the T cell receptor (TCR) activated ZAP-70, Ras, Raf, Erk and Elk (TCF) pathway. Development of a rapid purification method for large quantities of Ara h 2, identification of both the B and T cell epitopes of this allergen, as well as establishment of the signal transduction pathway will be useful in the evolution of diagnostic tools and in the design of novel peptide-mediated immunotherapies for treatment of peanut allergies.

Introduction

Peanuts have historically attracted interest as a potential source of proteins for umans as well as animal consumption because of the projected shortage of food oteins throughout the world. In the past decade there has been an increase in lergic reactions to peanut proteins. Various minor allergen and three major peanut lergens and have been identified among which Ara h 2 is one of the most nportant. Unlike the food hypersensitivity reactions to milk and eggs, peanut /persensitivity reactions usually persist into adulthood and last for a lifetime. In ddition, hypersensitivity reactions to peanuts tend to be more severe than those to her food allergens. Several reports have detailed fatal and near-fatal anaphylactic eactions occurring in adolescents and adults following the ingestion of peanuts or eanut products.

The development of an IgE response to an allergen involves a series of teractions between antigen-presenting cells (APCs), T cells, and B cells. Initially PCs present small peptide fragments (T cell epitopes) in conjunction with MHC class molecules to T cells. T cells bearing the appropriate complementary T cell eceptor (TCR) will bind to the peptide MHC complex leading to further cognate teractions that result in the generation of a "second" signal, T-cell proliferation and /tokine generation (Th_2-like lymphocyte activation). The orderly sequence of events ecessary to produce allergen-specific IgE, and the critical role T-cells play in this rocess, has been studied in a variety of aeroallergens However, the role of T mphocytes and antigen specificity in the induction and regulation of the food allergic esponse is less well defined. Here, we characterize some of the immune responses o a major food allergen.

Fig. 1A.

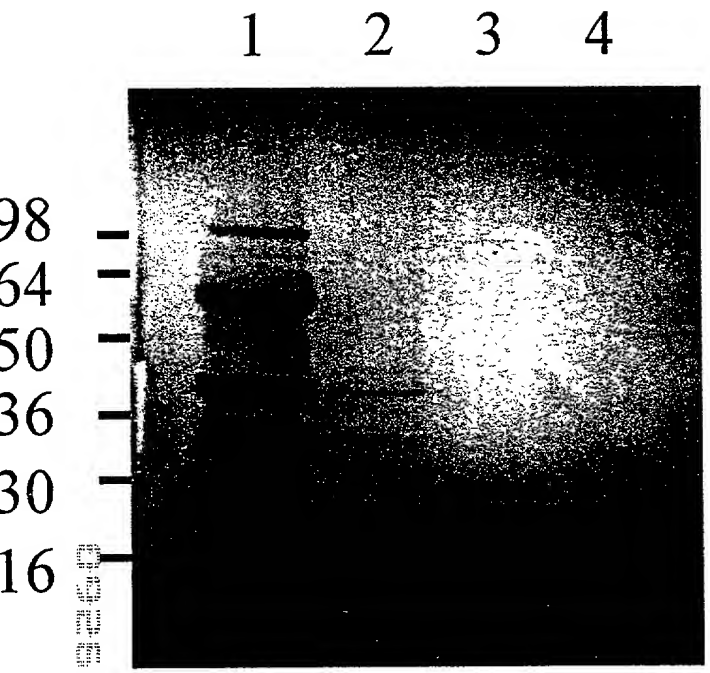

Fig 1.B.

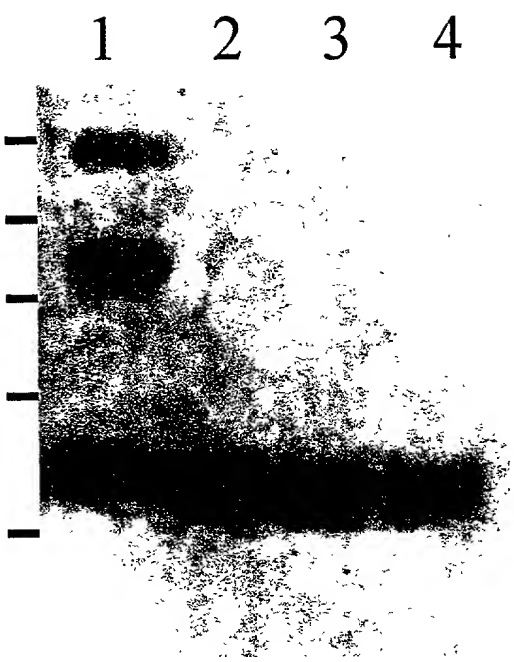

Table 12 Ara h 2 purification from crude peanut extracts.

	Total protein (mg)	Ara h 2 (Mg)	Fold Enrichment	% Yield
Crude lysate	20,000	500	-----	-----
$(NH_4)_2SO_4$	500	200	16	40
High prep DEAE	150	115	31	23
Phenyl Sepharose	77	75	39	15

NH3-LTILVALALFLLAAHASA**RQQWELQGDRRCQSQ**LERAN**LRPCEQHLMQKIQRD**

EDSYERDPYSPSQDPYSPSPYDRRGAGSSQ**HQERCCNELN**EFENN**QRCMCEALQQ**IM

ENQSDRLQGRQQEQQFKRELRNL**PQQCGLRAPQRCDLD**VESGGRDY-COOH

Table 13. Ara h 2 B-cell and T-cell Epitopes

B-cell Epitope Number	AA Sequence	Ara h 2 Position
3	DRRCQSQLER	27-36
6	YERDPYSPSQ	57-66
7	SQDPYSPSPY	65-74
T-cell epitope number	AA Sequence	Ara h 2 Position
1	RQQWELQGDRRCQSQ	19-33
2	LRPCEQHLMQKIQRDEDSYE	39-58
3	HQERCCNELN	84-93
4	QRCMCEALQQ	99-109
5	PQQCGLRAPQ	135-148

T-cell epitopes

B-cell epitopes

Summary & Conclusions

1. Peanut specific T-cell lines are primarily $CD4^+$

2. Peanut specific T-cell lines are stimulated through the T-cell receptor and the activation of the Ras, Raf, Zap-70, ERK, ELK-1 signal transduction pathway.

3. There are at least five T-cell epitopes on the major peanut allergen Ara h 2.

4. Most of the T-cell epitopes do not overlap with known IgE binding epitopes.

SEQUENCE LISTING

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(D) SOFTWARE: Word Perfect 6.0

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:

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(vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: US 60/077,763

(B) FILING DATE: 12 March 1998

(C) APPLICATION NUMBER:

(D) FILING DATE:

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(C) TELEX: Not applicable

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 23 amino acid residues

(B) TYPE: Amino acid sequence

(C) STRANDEDNESS: Not applicable

(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Peptide

(A) DESCRIPTION: 23 N-terminal amino acid residue sequence of a 14 kD protein/allergen isolated from a crude extract of peanuts (Arachis hypogaea L.) identified as Ara h 3 (IUIS/WHO)

(iii) HYPOTHETICAL: No

(iv) ANTI-SENSE: Not applicable

(v) FRAGMENT TYPE: N-terminal fragment

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Arachis hypogaea L.

(B) STRAIN: Southeastern runners

(C) INDIVIDUAL ISOLATE: Commercial lots North Carolina State Univ.

(D) DEVELOPMENT STAGE: Mature raw peanuts

(E) HAPLOTYPE: Not applicable

(F) TISSUE TYPE: Mature peanuts

(G) CELL TYPE: Not applicable

(H) CELL LINE: Not applicable

(I) ORGANELLE: Not applicable.

(vii) IMMEDIATE SOURCE: Crude soluble whole peanut extract

(A) LIBRARY: Not applicable

(B) CLONE: Not applicable

(viii) POSITION IN GENOME: Not applicable

(ix) FEATURE:

(A) NAME/KEY:

(B) LOCATION: Not registered

(C) IDENTIFICATION METHOD: Not completed

(D) OTHER INFORMATION: Ara h 3 allergen isolated from crude extract of Arachis hypogaea L with an apparent molecular wt of 14 kD that binds to IgE in human serum from patients with peanut immediate hypersensitivity

(x) PUBLICATION INFORMATION:

(A) AUTHORS:

(B) TITLE:

(C) JOURNAL:

(D) VOLUME:

(E) ISSUE:

(F) PAGES:

(G) DATE:

(H) DOCUMENT NUMBER:

(I) FILING DATE:

(J) PUBLICATION DATE:

(K) RELEVANT RESIDUES:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

Ile Ser Phe Arg Gln Gln Pro Glu Glu Asn Ala X Gln Phe
1 5 10
Arg Leu Asn Ala Gln Arg Pro Asp
20

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 15 amino acid residues

(B) TYPE: Amino acid sequence.

(C) STRANDEDNESS: Not applicable

(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Peptide

(A) DESCRIPTION: 15 N-terminal amino acid residue sequence of a 15 kD protein/allergen isolated from a crude extract of peanuts (Arachis hypogaea L.) identified as Ara h 4 (IUIS/WHO)

(iii) HYPOTHETICAL: No

(iv) ANTI-SENSE: Not applicable

(v) FRAGMENT TYPE: N-terminal fragment

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Arachis hypogaea L.

(B) STRAIN: Southeastern runners

(C) INDIVIDUAL ISOLATE: Commercial lots North Carolina State Univ.

(D) DEVELOPMENT STAGE: Mature raw peanuts

(E) HAPLOTYPE: Not applicable

(F) TISSUE TYPE: Mature peanuts

(G) CELL TYPE: Not applicable

(H) CELL LINE: Not applicable

(I) ORGANELLE: Not applicable

(vii) IMMEDIATE SOURCE: Crude soluble whole peanut extract

(A) LIBRARY: Not applicable

(B) CLONE: Not applicable

(viii) POSITION IN GENOME: Not applicable

(ix) FEATURE:

(A) NAME/KEY:

(B) LOCATION: Not registered

(C) IDENTIFICATION METHOD: Not completed

(D) OTHER INFORMATION: Ara h 4 allergen isolated from crude extract of Arachis hypogaea L with an apparent molecular wt of 15 kD that binds to IgE in human serum from patients with peanut immediate hypersensitivity

///

(x) PUBLICATION INFORMATION:

(A) AUTHORS:

(B) TITLE:

(C) JOURNAL:

(D) VOLUME:

(E) ISSUE:

(F) PAGES:

(G) DATE:

(H) DOCUMENT NUMBER:

(I) FILING DATE:

(J) PUBLICATION DATE:

(K) RELEVANT RESIDUES:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Arg	Glu	Phe	Ser	X	Glu	Gly	Glu	His	Gly	Arg	Arg	Glu	Glu
1						5						10	

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 amino acid residues

(B) TYPE: Amino acid sequence

(C) STRANDEDNESS: Not applicable

(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Peptide

(A) DESCRIPTION: 20 N-terminal amino acid residue sequence of a 10 kD protein/allergen (lower band) isolated from a crud extract of peanuts (Arachis hypogaea L.) identified as Ara h 5 (IUIS/WHO)

(iii) HYPOTHETICAL: No

(iv) ANTI-SENSE: Not applicable

(v) FRAGMENT TYPE: N-terminal fragment

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Arachis hypogaea L.

(B) STRAIN: Southeastern runners

(C) INDIVIDUAL ISOLATE: Commercial lots North Carolina State Univ.

(D) DEVELOPMENT STAGE: Mature raw peanuts

(E) HAPLOTYPE: Not applicable

(F) TISSUE TYPE: Mature peanuts

(G) CELL TYPE: Not applicable

(H) CELL LINE: Not applicable

(I) ORGANELLE: Not applicable

(vii) IMMEDIATE SOURCE: Crude soluble whole peanut extract

(A) LIBRARY: Not applicable

(B) CLONE: Not applicable

(viii) POSITION IN GENOME: Not applicable

(ix) FEATURE:

(A) NAME/KEY:

(B) LOCATION: Not registered

(C) IDENTIFICATION METHOD: Not completed

(D) OTHER INFORMATION: Ara h 5 allergen isolated from crude extract of Arachis hypogaea L with an apparent molecular wt of 10 kD that binds to IgE in human serum from patients with peanut immediate hypersensitivity

(x) PUBLICATION INFORMATION:

(A) AUTHORS:

(B) TITLE:

(C) JOURNAL:

(D) VOLUME:

(E) ISSUE:

(F) PAGES:

(G) DATE:

(H) DOCUMENT NUMBER:

(I) FILING DATE:

(J) PUBLICATION DATE:

(K) RELEVANT RESIDUES:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

Ser Thr Arg Ser Ser Asp Gln Gln Gln Arg X X Asp Glu Le
1 5 10
Asn Glu Met X Asn
20

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:

(B) TYPE: Amino acid sequence

(C) STRANDEDNESS: Not applicable

(D) TOPOLOGY: Unknown

(ii) MOLECULE TYPE: Glycoprotein

(A) DESCRIPTION:

Ara h 3 (IUIS/WHO nomenclature)

(iii) HYPOTHETICAL: No

(iv) ANTI-SENSE: Not applicable

(v) FRAGMENT TYPE: N-terminal fragment

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Arachis hypogaea L.

(B) STRAIN: Southeastern runners

(C) INDIVIDUAL ISOLATE: Commercial lots North Carolina State Univ.

(D) DEVELOPMENT STAGE: Mature raw peanuts

(E) HAPLOTYPE: Not applicable

(F) TISSUE TYPE: Mature peanuts

(G) CELL TYPE: Not applicable

(H) CELL LINE: Not applicable

(I) ORGANELLE: Not applicable

(vii) IMMEDIATE SOURCE: Crude soluble whole peanut extract

(A) LIBRARY: Not applicable

(B) CLONE: Not applicable

(viii) POSITION IN GENOME: Not applicable

(ix) FEATURE:

(A) NAME/KEY:

(B) LOCATION: Not registered

(C) IDENTIFICATION METHOD: Not completed

(D) OTHER INFORMATION: Ara h 3 allergen isolated from crude extract of Arachis hypogaea L

(x) PUBLICATION INFORMATION:

(A) AUTHORS:

(B) TITLE:

(C) JOURNAL:

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(H) DOCUMENT NUMBER:

(I) FILING DATE:

(J) PUBLICATION DATE:

(K) RELEVANT RESIDUES:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

```
1    I   S   F   R   Q   Q   P   E   E   N   A   C   Q   F   Q   R   L   N   A
         CGG CAG CAA CCG GAG GAG AAC GCG TGC CAG TTC CAG CGC CTC AAT GCG C

25   R   I   E   S   E   G   G   Y   I   E   T   W   N   P   N   N   Q   E   F
   CGC ATT GAA TCA GAG GGC GGT TAC ATT GAG ACT TGG AAC CCC AAC AAC CAG GAG TTC G

49   A   L   S   R   L   V   L   R   R   N   A   L   R   R   P   F   Y   S   N
   GCC CTC TCT CGC TTA GTC CTC CGC CGC AAC GCC CTT CGT AGG CCT TTC TAC TCC AAT G

73   F   I   Q   Q   G   R   G   Y   F   G   L   I   F   P   G   C   P   R   H
   TTC ATC CAG CAA GGA AGG GGA TAC TTT GGG TTG ATA TTC CCT GGT TGT CCT AGA CAC T

97   T   Q   G   R   R   S   Q   S   Q   R   P   P   R   R   L   Q   G   E   D
   ACA CAA GGT CGT CGA TCT CAG TCC CAA AGA CCA CCA AGA CGT CTC CAA GGA GAA GAC C

121  R   D   S   H   Q   K   V   H   R   F   D   E   G   D   L   I   A   V   P
     CGA GAT AGT CAC CAG AAG GTG CAC CGT TTC GAT GAG GGT GAT CTC ATT GCA GTT CCC

145  W   L   Y   N   D   H   D   T   D   V   V   A   V   S   L   T   D   T   N
     TGG CTC TAC AAC GAC CAC GAC ACT GAT GTT GTT GCT GTT TCT CTT ACT GAC ACC AAC

169  L   D   Q   F   P   R   R   F   N   L   A   G   N   T   E   Q   E   F   L
```

```
    CTT GAT CAG TTC CCC AGG AGA TTC AAT TTG GCT GGG AAC ACG GAG CAA GAG TTC TTA

193  S   R   Q   S   R   R   R   S   L   P   Y   S   P   Y   S   P   Q   S   Q
    AGC AGA CAA AGC AGA CGA AGA AGC TTA CCA TAT AGC CCA TAC AGC CCG CAA AGT CAG

217  R   E   F   S   P   R   G   Q   H   S   R   R   E   R   A   G   Q   E   E
    CGT GAA TTT AGC CCT CGA GGA CAG CAC AGC CGC AGA GAA CGA GCA GGA CAA GAA GAA

241  N   I   F   S   G   F   T   P   E   F   L   E   Q   A   F   Q   V   D   D
    AAC ATC TTC AGC GGC TTC ACG CCG GAG TTC CTG GAA CAA GCC TTC CAG GTT GAC GAC

265  N   L   R   G   E   T   E   S   E   E   E   G   A   I   V   T   V   R   G
    AAC CTA AGA GGC GAG ACC GAG AGT GAA GAA GAG GGA GCC ATT GTG ACA GTG AGG GGA

289  S   P   D   R   K   R   R   A   D   E   E   E   E   Y   D   E   D   E   Y
    AGC CCA GAT AGA AAG AGA CGT GCC GAC GAA GAA GAG GAA TAC GAT GAA GAT GAA TAT

313  D   R   R   R   G   R   G   S   R   G   R   G   N   G   I   E   E   T   I
    GAT AGA AGG CGT GGC AGG GGA AGC AGA GGC AGG GGG AAT GGT ATT GAA GAG ACG ATC

337  K   K   N   I   G   R   N   R   S   P   D   I   Y   N   P   Q   A   G   S
    AAA AAG AAC ATT GGT AGA AAC AGA TCC CCT GAC ATC TAC AAC CCT CAA GCT GGT TCA

361  D   L   N   L   L   I   L   R   W   L   G   P   S   A   E   Y   G   N   L
    GAT CTC AAC CTT CTA ATA CTT AGG TGG CTT GGA CCT AGT GCT GAA TAT GGA AAT CTC

385  F   V   A   H   Y   N   T   N   A   H   S   I   I   Y   R   L   R   G   R
    TTT GTC GCT CAC TAC AAC ACC AAC GCA CAC AGC ATC ATA TAT CGA TTG AGG GGA CGG

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    GTG GAC AGC AAC GGC AAC AGA GTG TAC GAC GAG GAG CTT CAA GAG GGT CAC GTG CTT

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    ATA GCC AAC CTC GCC GGT GAA AAC TCC GTC ATA GAT AAC CTG CCG GAG GAG GTG GTT

481  L   Q   R   E   Q   A   R   Q   L   K   N   N   N   P   F   K   F   F   V
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505  S   P   R   A   V   A   *
    TCT CCG AGG GCT GTG GCT TAA
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Ala Leu Ser Arg Leu Val Leu Arg Arg Asn Ala Leu Arg Arg Pro Phe
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Phe Gly Leu Ile Phe Pro Gly Cys Pro Arg His Tyr Glu Glu Pro His
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Glu Glu Glu Gly Ala Ile Val Thr Val Arg Gly Gly Leu Arg Ile Leu
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Ser Pro Asp Arg Lys Arg Arg Ala Asp Glu Glu Glu Glu Tyr Asp Glu
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Asp Glu Tyr Glu Tyr Asp Glu Glu Asp Arg Arg Arg Gly Arg Gly Ser
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Arg Gly Arg Gly Asn Gly Ile Glu Glu Thr Ile Cys Thr Ala Ser Ala
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Lys Lys Asn Ile Gly Arg Asn Arg Ser Pro Asp Ile Tyr Asn Pro Gln
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Ala Gly Ser Leu Lys Thr Ala Asn Asp Leu Asn Leu Leu Ile Leu Arg
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Trp Leu Gly Leu Ser Ala Glu Tyr Gly Asn Leu Tyr Arg Asn Ala Leu
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Phe Val Ala His Tyr Asn Thr Asn Ala His Ser Ile Ile Tyr Arg Leu
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Alignment Of The Primary Amino Acid Sequences Of Ara h 1
And Phaseolin A Chain

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VLGLTFSGSGDEVMKLINKQSGSYFVDAH

CLAIM OR CLAIMS

What is claimed is:

1. A peanut allergen having the tertiary structure shown and described herein.

2. The peanut allergen as recited in claim 1, wherein said peanut allergen is peanut allergen Ara h 1.

3. The peanut allergen as described in claim 2, wherein said tertiary structure consists of two sets of opposing sets of anti-parallel β-sheets in swiss roll topology with the terminal regions of the molecule consisting of α-helical bundles containing three helices apiece.

4. The peanut allergen as recited in claim 3, wherein there are numerous protease digestion sites throughout the length of this protein and the structure is so compact that potential cleavage sites are inaccessible until the protein is denatured.

5. The peanut allergen as recited in claim 4, wherein the formation of a trimeric complex and further higher order aggregation affords the molecule protection from protease digestion and denaturation and allows passage of the protein across the small intestine.

6. The peanut allergen as recited in claim 3, wherein the Ara h 1 IgE-binding epitopes are clustered on the surface of the molecule.

7. A tertiary structure for a peanut allergen Ara h 1 having the structure as shown and described herein.

8. The tertiary structure as recited in claim 7, wherein said structure is shown in at least one of Figures 3, 5, 11, 12, 31, 32, 38, 47, 48, and 49.

9. A method of producing the tertiary structure as recited in claim 7.

10. A method of using the nucleic acid sequences, proteins and peptides as shown and described herein.

11. Therapeutic compositions comprising proteins, nucleic acid molecules, antibodies, and

the like as shown and described herein.

12. The use of the therapeutic compositions of claim 11, to protect a host animal from allergic reaction.

13. A modified allergen epitope having at least one of the hydrophobic amino acids located in the middle of the epitope modified to prevent IgE binding.

14. The IgE binding peptides described in Table 1.

15. The alignment of the primary amino acid sequences of Ara h 1 and phaseolin A chain as shown in Table 3.

16. A DNA clone having homology with OP18, prothymosin alpha, and MM-1.

17. T-cell epitopes of Ara h 2 as shown and described herein.

18. The T-cell epitopes as recited in claim 17, wherein said epitopes are identified as epitope 1 (AA18-28), epitope 2 (AA45-55), epitope 3 (AA95-108), and epitope 4 (AA134-144).

19. The epitopes as recited in claim 18, wherein epitopes 1, 2 and 4 have overlapping sequences with Ara h 2 B-cell epitopes, whereas epitope 3 does not overlap IgE-binding epitopes.

20. A non-anaphylactic, T-cell directed immunotherapeutic peptide based on the epitopes of claim 18.

21. A peanut allergen Ara h 3 identified by using soy-absorbed serum IgE antibodies from peanut sensitive individuals and having significant sequence homology with the glycine family of seed storage proteins.

22. IgE-binding regions unique to soybean positive IgE binding, unique to peanut positive IgE binding, and common to both peanut and soybean IgE-binding regions as shown and

described herein.

23. An isolated recombinant peanut allergen designated Ara h 3.

24. An isolated nucleotide molecule encoding the peanut allergen designated Ara h 3.

25. A mutated peanut allergen protein having one or more IgE binding epitopes modified to reduce binding to IgE.

26. A method of treating an individual to reduce the clinical response to an allergen comprising administering to the individual a modified allergen which is less reactive with IgE in an amount and for a time sufficient to reduce the allergic reaction to the unmodified allergen.

TERTIARY STRUCTURE OF PEANUT ALLERGEN ARA H 1

ABSTRACT OF THE DISCLOSURE

Allergy to peanut is a significant health problem because of the prevalence and potential severity of the reaction. Ara h 1, a major peanut allergen, has been isolated and characterized and was shown to consist of 626 amino acids and contain 23 linear IgE-binding epitopes, 6-10 residues in length. The amino acids important for peanut-specific IgE binding were determined by synthesizing wild type and mutant peptides with single alanine, glycine or methionine substitutions at each position followed by incubation in pooled serum from patients with peanut hypersensitivity. From this analysis it was determined that amino acids which reside in the midele of the epitope were generally more critical for IgE binding. Furthermore, though polar charged residues occur most frequently within the epitopes. apolar residues were found to be more important for IgE binding. In addition, it was found that each epitope could be mutated resulting in loss of ability to bind IgE with only a single amino acid substitution. To further characterize the epitopes a homology-based molecular model of the Ara h 1 protein was made. The model represents residues 171-586 allowing visualization of epitopes 10-22. The majority of these epitopes appear to be clustered to certain areas of the molecule. Many of the critical amino acids involved in binding are evenly distributed on the surface and not buried in the hydrophobic core. The information from the mutational analysis along with the molecular model will aid in the design of immunotherapies.

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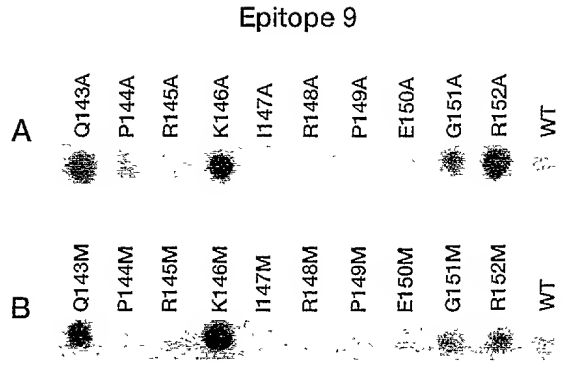

FIG. 1. **Single amino acid changes to epitope 9 result in loss of IgE binding to this epitope.** Epitope 9 was synthesized with an alanine (*Panel A*) or methionine (*Panel B*) residue substituted for one of the amino acids and probed with a pool of serum IgE from 15 patients with documented peanut hypersensitivity. The *letters* across the *top* of each *panel* indicate the one-letter amino acid code for the residue normally at the position and the amino acid that was substituted for this residue. The *numbers* indicate the position of each residue in the Ara h1 protein. *WT*, indicates the wild type peptide (no amino acid substitutions).

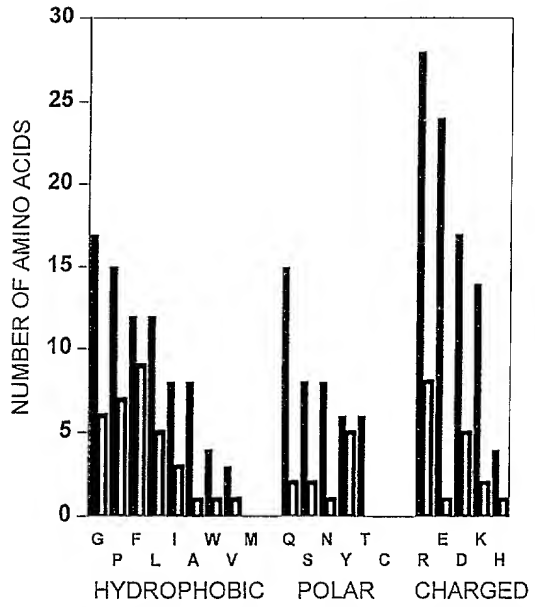

FIG. 2. **Hydrophobic amino acids are more critical to IgE binding.** The type of each amino acid within the Ara h1 epitopes was assessed relative to its importance to IgE binding. The *closed boxes* represent the total number of a particular type of amino acid residue found in all of the Ara h1 epitopes, whereas the *open boxes* represent the number of that type of residue which, when replaced, was found to result in the loss of IgE binding.

A

```
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B

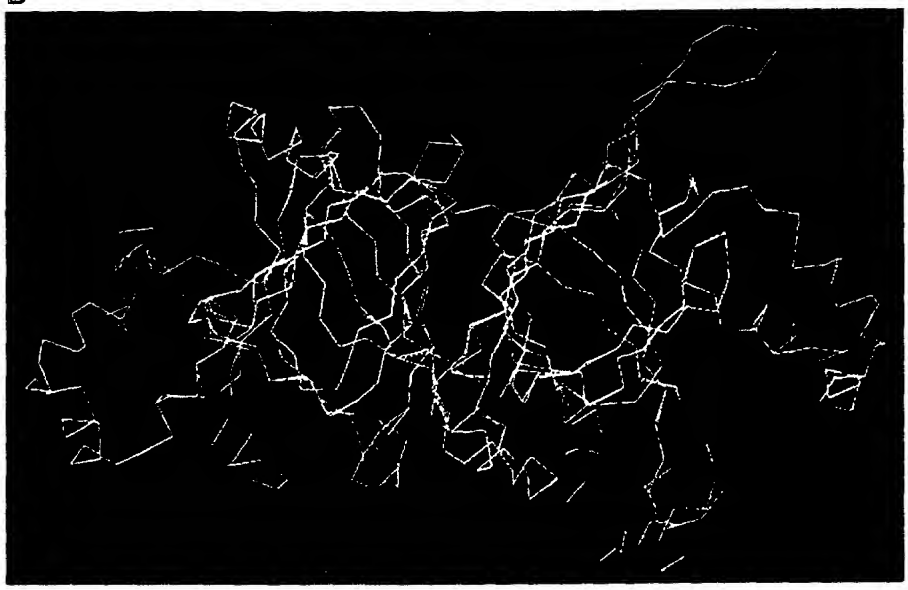

FIG. 3. **Alignment of the primary amino acid sequences and the α-carbon structural alignment of Ara h1 and the phaseolin A chain.** *Panel A* represents the single letter amino acid code for Ara h1 residues 172–586 (*top line*) and all of the phaseolin A chain (*bottom line*). The structurally conserved regions, shown in *bold type*, were used to develop the initial backbone of the Ara h1 model. The other regions were used in protein loop searches to complete the tertiary structure of Ara h1. *Panel B* represents the α-carbon alignment of the final model of Ara h1 (*white*) *versus* the phaseolin A chain (*yellow*). Labeled residues Asn^{1} and Arg^{415} represent the N and C termini of the Ara h1 model, respectively. Areas between labeled amino acids Asn^{169}-Val^{193}, Val^{212}-Gly^{221}, Phe^{240}-Pro^{226}, Pro^{226}-Phe^{240} and Arg^{300}-Asn^{323} represent areas of structural uncertainty due to insertions in Ara h1 or unsolved sequences in phaseolin. Note that the residue numbers are shifted due to the N-terminal deletion from the Ara h1 coding sequence found in the GenBank™ data bank (the amino acid sequence of this protein can be accessed through the GenBank™ data bank under GenBank Accession Number L34402 (17)).

Phi/Psi Plot

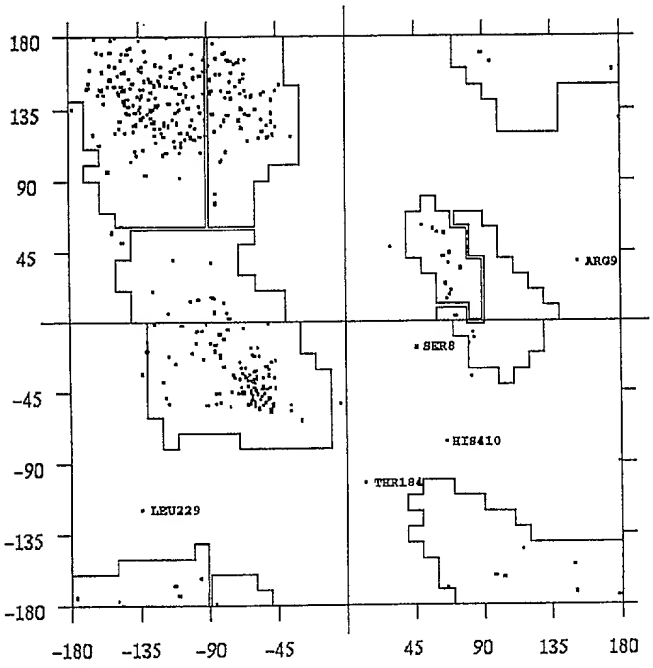

FIG. 4. **Most of the ϕ/ψ torsion angles of the amino acid residues in the Ara h1 tertairy structure model are allowed.** A plot of the ϕ and ψ angles for the amino acids in the Ara h1 tertiary structure model is shown. Each *dot within* one of the *boxes* represents an amino acid that has acceptable torsion angles. Major outliers are indicated by their three letter amino acid code and position using the N-terminal as residue 1 as in Fig. 3.

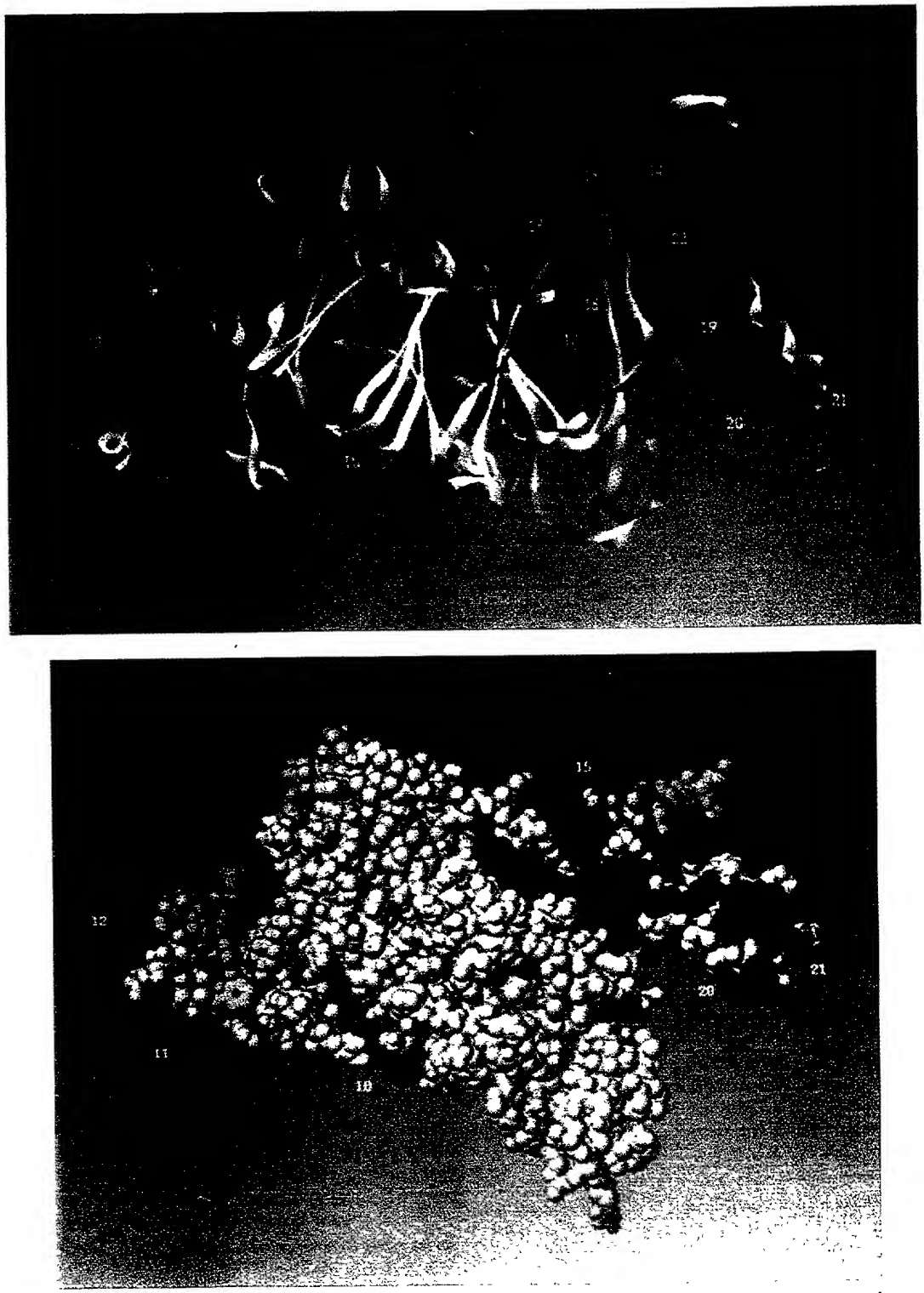

FIG. 5. **The majority of the Ara h1 IgE binding epitopes are clustered in two regions of the allergen.** The *top panel* represents a ribbon diagram of Ara h1 tertiary structure. The *numbered red areas* are IgE binding epitopes 10–22. Epitopes 13 and portions of 14 and 15 lie in an area of structural uncertainty. The *bottom panel* is a space filling model of Ara h1 tertiary structure. The *red* areas represent the IgE binding epitopes, and the *yellow atoms* are the residues that were determined to be critical for IgE binding to occur.

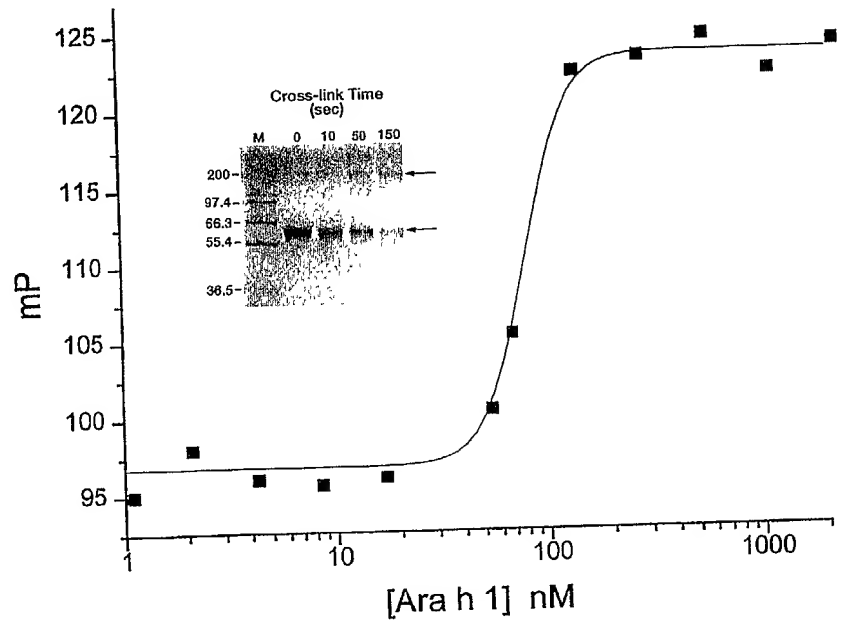

FIG. 6. **The Ara h1 allergen forms a stable trimeric structure.** Trace fluorescein-labeled Ara h1 was mixed with unlabeled Ara h1, and fluorescence polarization measurements (*mP*) were made at each concentration. Each point represents the average of three different experiments. Samples from the 200 nM concentration were then subjected to cross-linking with constant amounts of DSP for varying lengths of time, and the products were electrophoresed on SDS-polyacrylamide gels. Protein bands were visualized by Coomassie staining. *Lower arrow* indicates the Ara h1 monomer (~60 kDa), and the *upper band* represents the Ara h1 trimer (~180 kDa).

CHYMOTRYPSIN

Panel. A.

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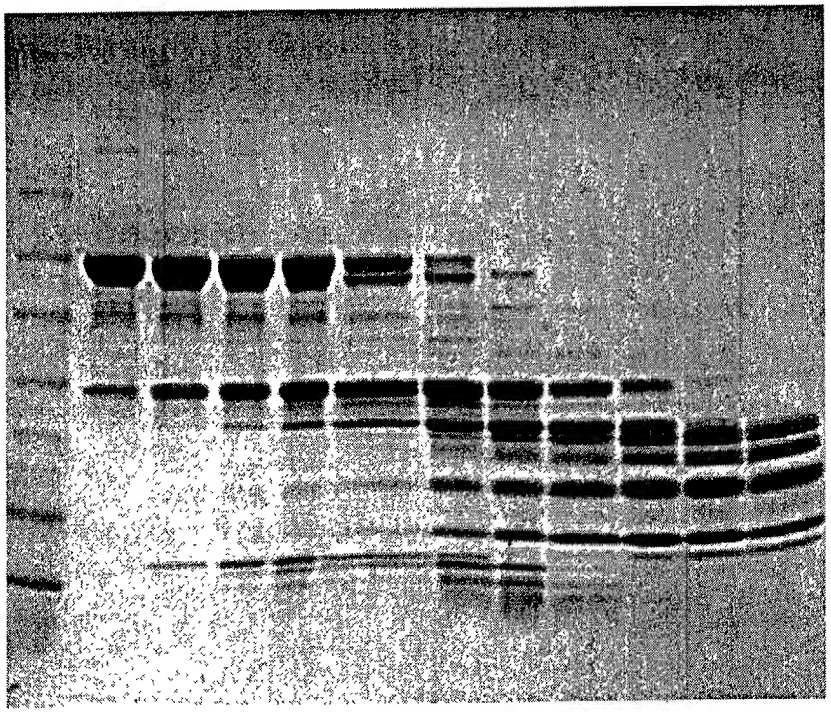

Panel. B.

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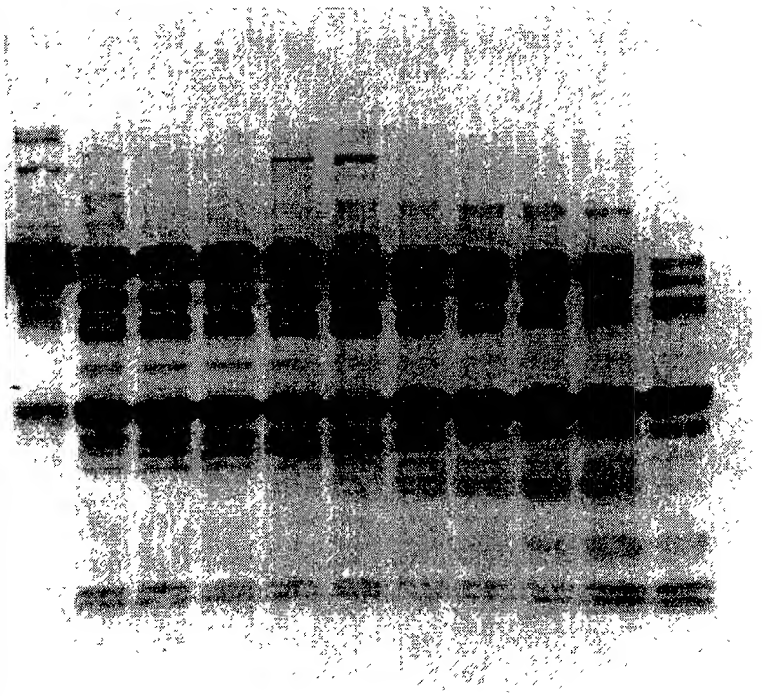

Fig. 7 (A, B)

CHYMOTRYPSIN

Panel C.

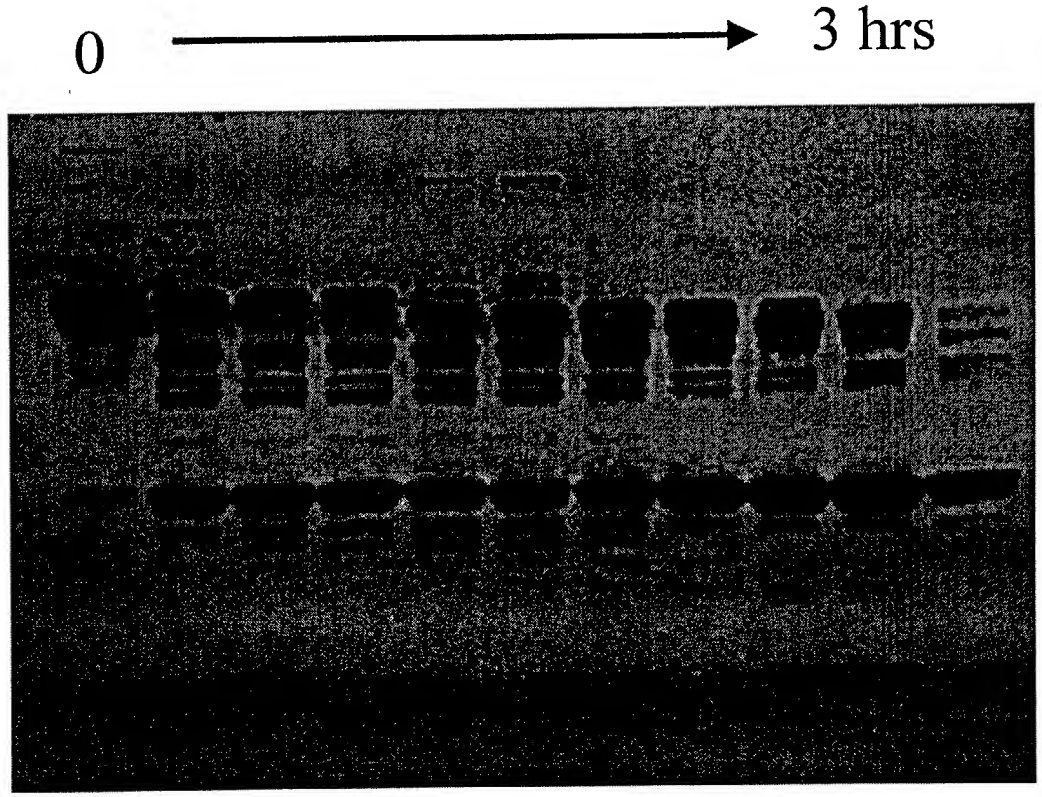

Fig. 7 (c)

TRYPSIN

Panel A.

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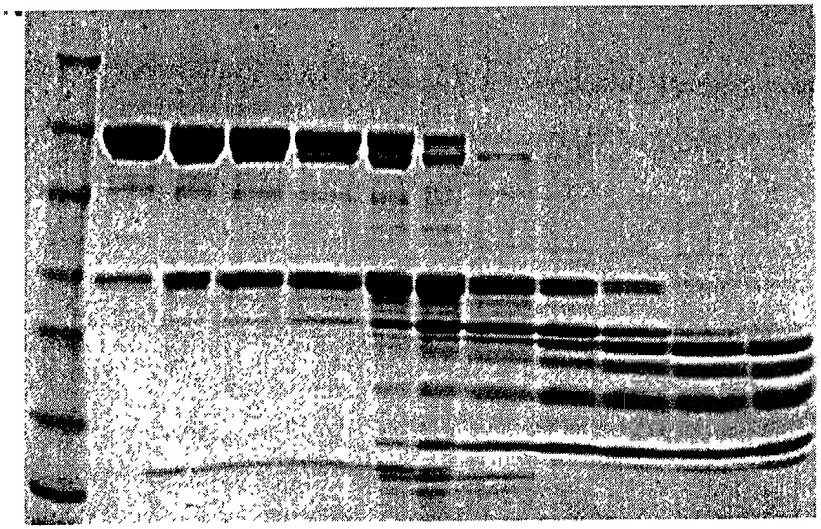


Panel B.

0 → 3 hrs

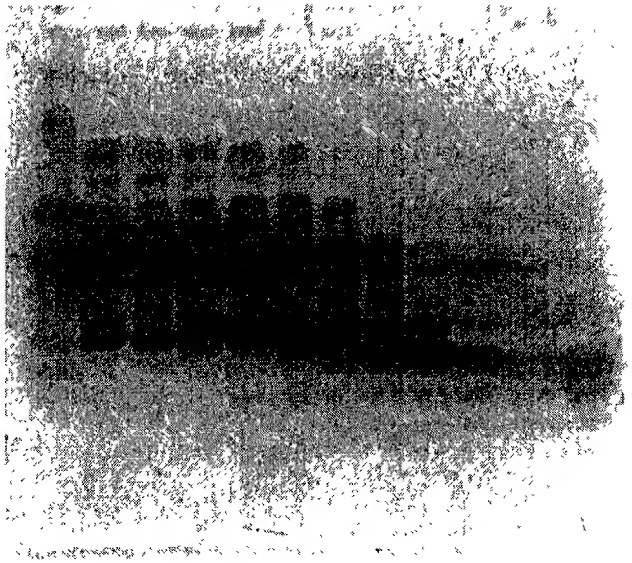


Fig. 8 (A, B)

TRYPSIN

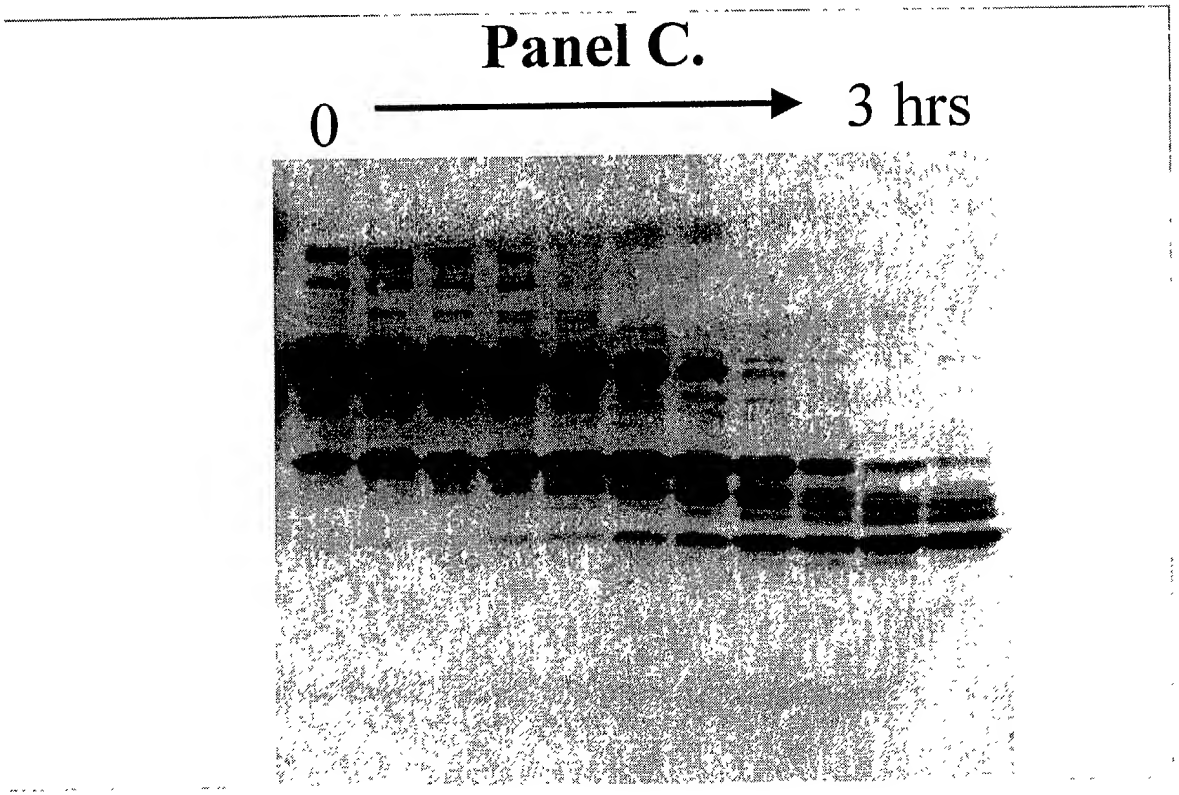


Fig. 8 (c)

Pepsin

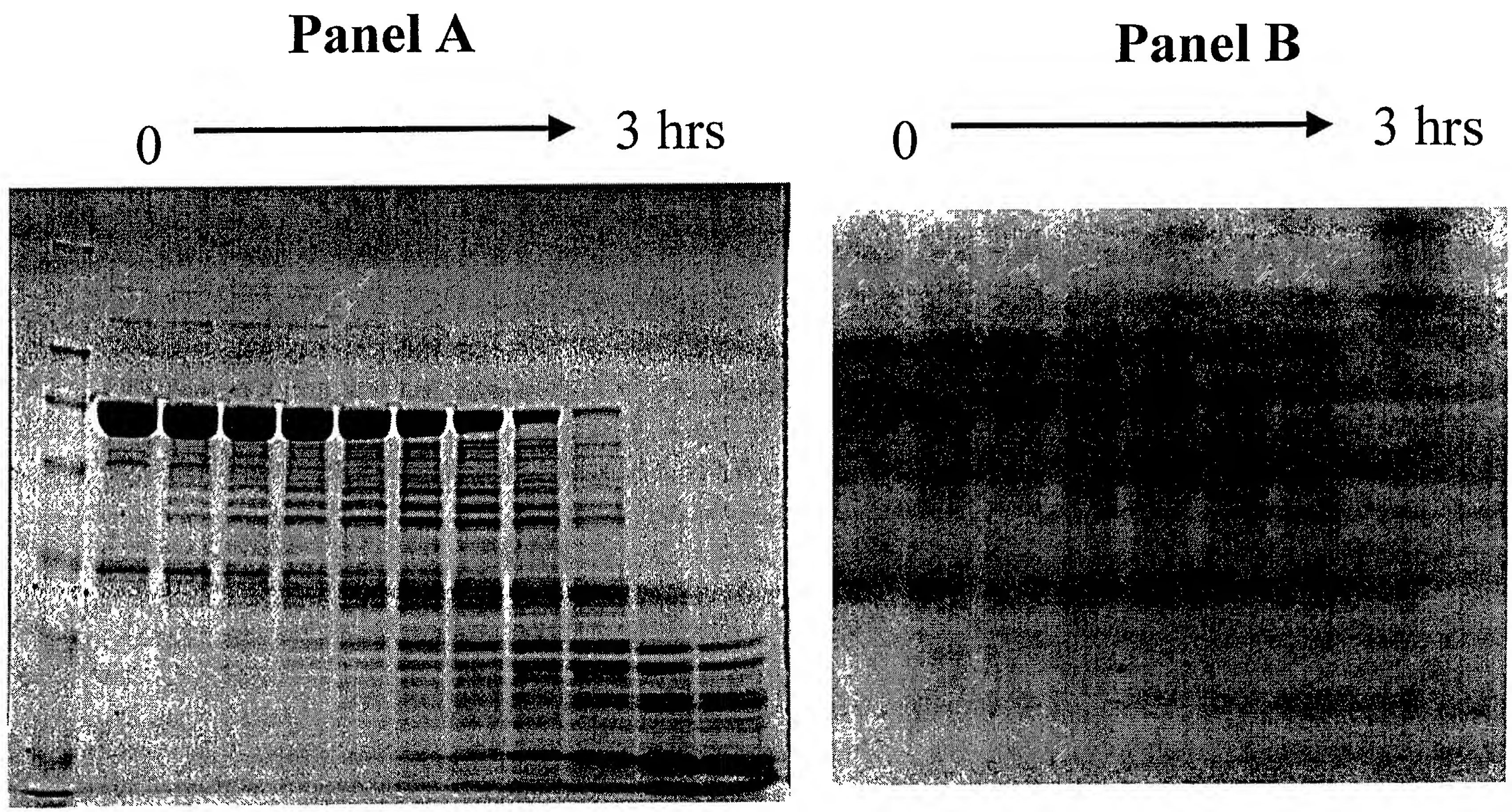


Fig. 9

MyoD Digestion

Fig. 10

Ara h 3 Amino Acid Sequence

1	I	S	F	R	Q	Q	P	E	E	N	A	C	Q	F	Q	R	L	N	A	Q	R	P	D	N	R	I	E
28	S	E	G	G	Y	I	E	T	W	N	P	N	N	Q	E	F	E	C	A	G	V	A	L	S	R	L	V
54	L	R	R	N	A	L	R	R	P	F	Y	S	N	A	P	Q	E	I	F	I	Q	Q	G	R	G	Y	F
82	G	L	I	F	P	G	C	P	R	H	Y	E	E	P	H	T	Q	G	R	R	S	Q	S	Q	R	P	P
109	R	R	L	Q	G	E	D	Q	S	Q	Q	Q	R	D	S	H	Q	K	V	H	R	F	D	E	G	D	L
136	I	A	V	P	T	G	V	A	F	W	L	Y	N	D	H	D	T	D	V	V	A	V	S	L	T	D	T
163	N	N	N	D	N	Q	L	D	Q	F	P	R	R	F	N	L	A	G	N	T	E	Q	E	F	E	F	L
190	R	Y	Q	Q	Q	S	R	Q	S	R	R	R	S	L	P	Y	S	P	Y	S	P	Q	S	Q	P	R	Q
207	E	E	R	E	F	S	P	R	G	Q	H	S	R	R	E	R	A	G	Q	E	E	E	N	E	G	G	N
234	I	F	S	G	F	T	P	E	F	L	E	Q	A	F	Q	V	D	D	R	Q	I	V	Q	N	L	R	G
261	E	T	E	S	E	E	E	G	A	I	V	T	V	R	G	G	L	R	I	L	S	P	D	R	K	R	R
288	A	D	E	E	E	E	Y	D	E	D	E	Y	E	Y	D	E	E	D	R	R	R	G	R	G	S	R	G
315	R	G	N	G	I	E	E	T	I	C	T	A	S	A	K	K	N	I	G	R	N	R	S	P	D	I	Y
342	N	P	Q	A	G	S	L	K	T	A	N	D	L	N	L	L	I	L	R	W	L	G	L	S	A	E	Y
369	G	N	L	Y	R	N	A	L	F	V	A	H	Y	N	T	N	A	H	S	I	I	Y	R	L	R	G	R
396	A	H	V	Q	V	V	D	S	N	G	N	R	V	Y	D	E	E	L	Q	E	G	H	V	L	V	V	P
423	Q	N	F	A	V	A	G	K	S	Q	S	E	N	F	E	Y	V	A	F	K	T	D	S	R	P	S	I
450	A	N	L	A	G	E	N	S	V	I	D	N	L	P	E	E	V	V	A	N	S	Y	G	L	Q	R	E
477	Q	A	R	Q	L	K	N	N	N	P	F	K	F	F	V	P	P	S	Q	Q	S	P	R	A	V	A	

Fig. 11

A.

```
          51                                                  100
Ara h 3   SEGGYIETWN PNNQEFECAG VALSRLVLRR NALRRPFYSN APQEIFIQQG
 G1 Soy   SEGGLIETWN PNNKPFQCAG VALSRCTLNR NALRRPSYTN GPQEIYIQQG
 G2 Soy   SEGGFIETWN PNNKPFQCAG VALSRCTLNR NALRRPSYTN GPQEIYIQQG
 A2 Pea   SEGGLIETWN PNNKQFRCAG VALSRATLQH NALRRPYYSN APQEIFIQQG

          101                                                 150
Ara h 3   RGYFGLIFPG CPRHYEEPHT QGRRSQSQRP PRRLQGEDQS QQQRDSHQKV
 G1 Soy   KGIFGMIYPG CPSTFEEPQQ PQQRGQSSRP .......... ...QDRHQKI
 G2 Soy   NGIFGMIFPG CPSTYQEPQE SQQRGRSQRP .......... ...QDRHQKV
 A2 Pea   NGYFGMVFPG CPETFEEPQE SEQ.GEGRRY .......... ...RDRHQKV
```

B.

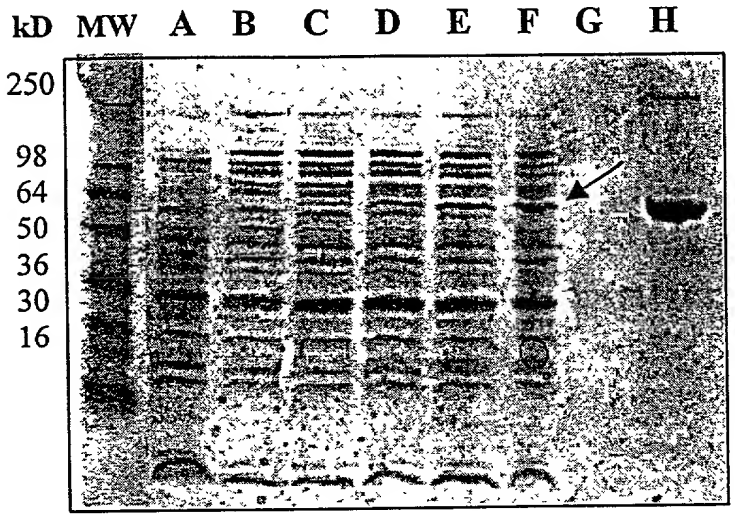
```
          351                                                 400
Ara h 3   EYDEDEY... .....EYDEE DRRRGRGSRG R......GNG IEETICTASA
 G1 Soy   EEEEDEKP.. ...QCKGKDK HCQRPRGSQS KSRR....NG IDETICTMRL
 G2 Soy   DDDEEEQP.. ...QCVETDK GCQR....QS KRSR....NG IDETICTMRL
 A2 Pea   DEDEERQPRH QRRRGEEEEE DKKERRGSQK GKSRRQGDNG LEETVCTAKL

          401                                                 450
Ara h 3   KKNIGRNRSP DIYNPQAGSL KTANDLNLLI LRWLGLSAEY GNLYRNALFV
 G1 Soy   RHNIGQTSSP DIYNPQAGSV TTATSLDFPA LSWLRLSAEF GSLRKNAMFV
 G2 Soy   RQNIGQNSSP DIYNPQAGSI TTATSLDFPA LWLLKLSAQY GSLRKNAMFV
 A2 Pea   RLNIGPSSSP DIYNPEAGRI KTVTSLDLPV LRWLKLSAEH GSLHKNAMFV

          451                                                 500
Ara h 3   AHYNTNAHSI IYRLRGRAHV QVVDSNGNRV YDEELQEGHV LVVPQNFAVA
 G1 Soy   PHYNLNANSI IYALNGRALI QVVNCNGERV FDGELQEGRV LIVPQNFVVA
 G2 Soy   PHYTLNANSI IYALNGRALV QVVNCNGERV FDGELQEGGV LIVPQNFAVA
 A2 Pea   PHYNLNANSI IYALKGRARL QVVNCNGNTV FDGELEAGRA LTVPQNYAVA
```

Fig. 12

A. *Bacterial Expression of Recombinant Ara h 3*



B. *Immunoblot Analysis of Total Bacterial Extract*

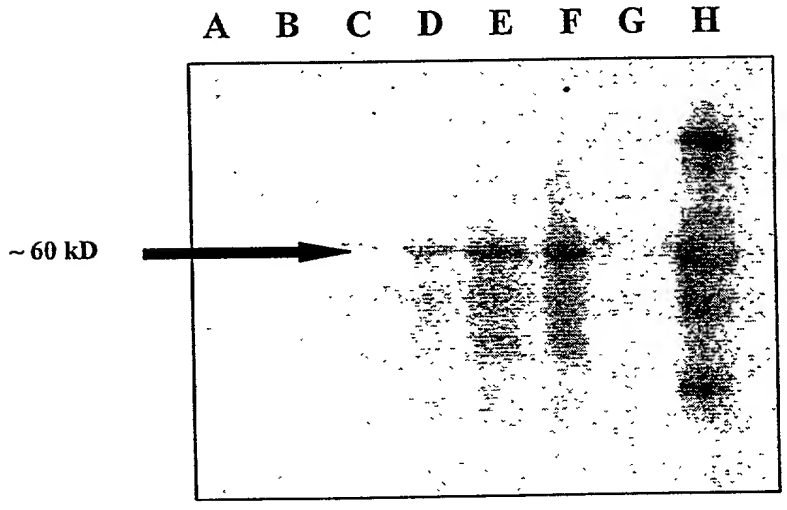
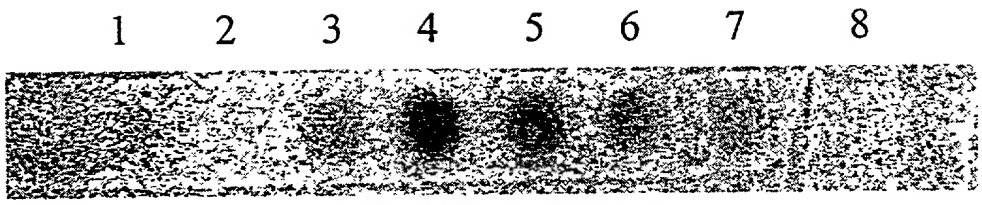


Fig. 13

A.


1 2 3 4 5 6 7 8

B.

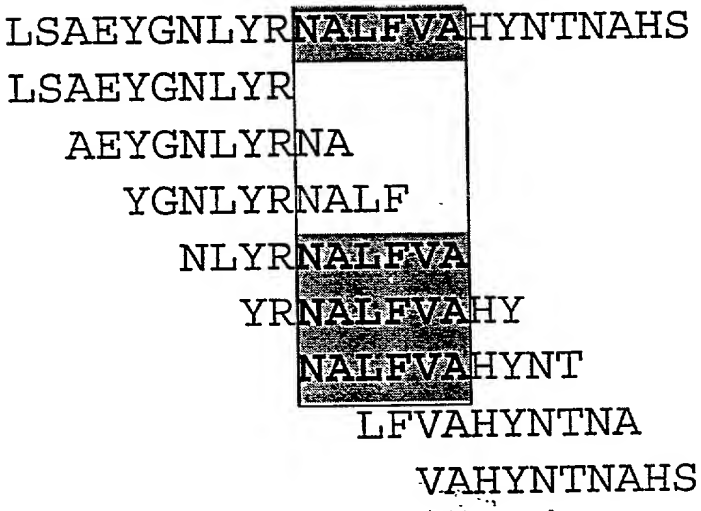
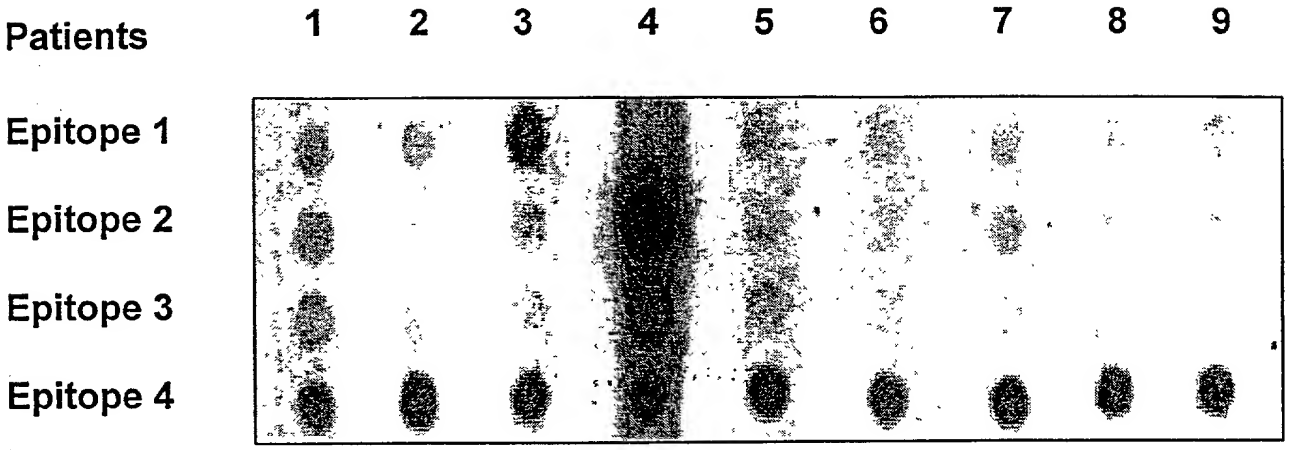

LSAEYGNLYRNALFVAHYNTNAHS
1. LSAEYGNLYR
2. AEYGNLYRNA
3. YGNLYRNALF
4. NLYRNALFVA
5. YRNALFVAHY
6. NALFVAHYNT
7. LFVAHYNTNA
8. VAHYNTNAHS

Fig. 14

A. *Epitope 4 is an Immunodominant Epitope*



B. *Percentage of Recognition for Each Epitope*

Epitope	Sequence	Position	Percentage
1	EQEFLRYQQQ	183-192	5% (1/20)
2	FTPEFLEQAF	246-255	25% (5/20)
3	EYEYDEEDRR	300-309	35% (7/20)
4	LYRNALFVAH	379-388	100%(20/20)

Fig. 15

Peptide 2

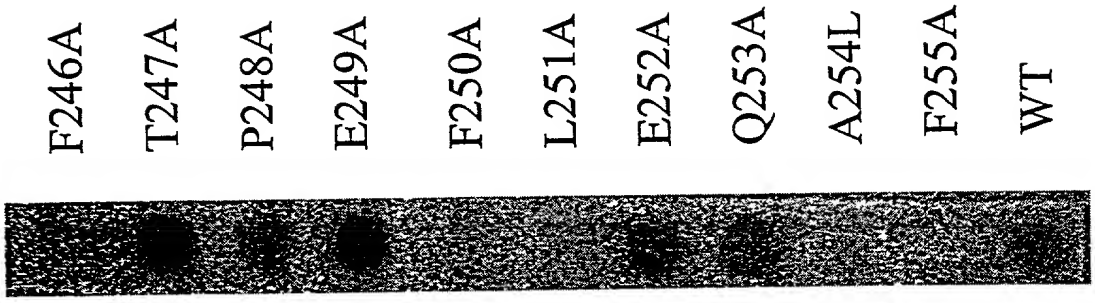
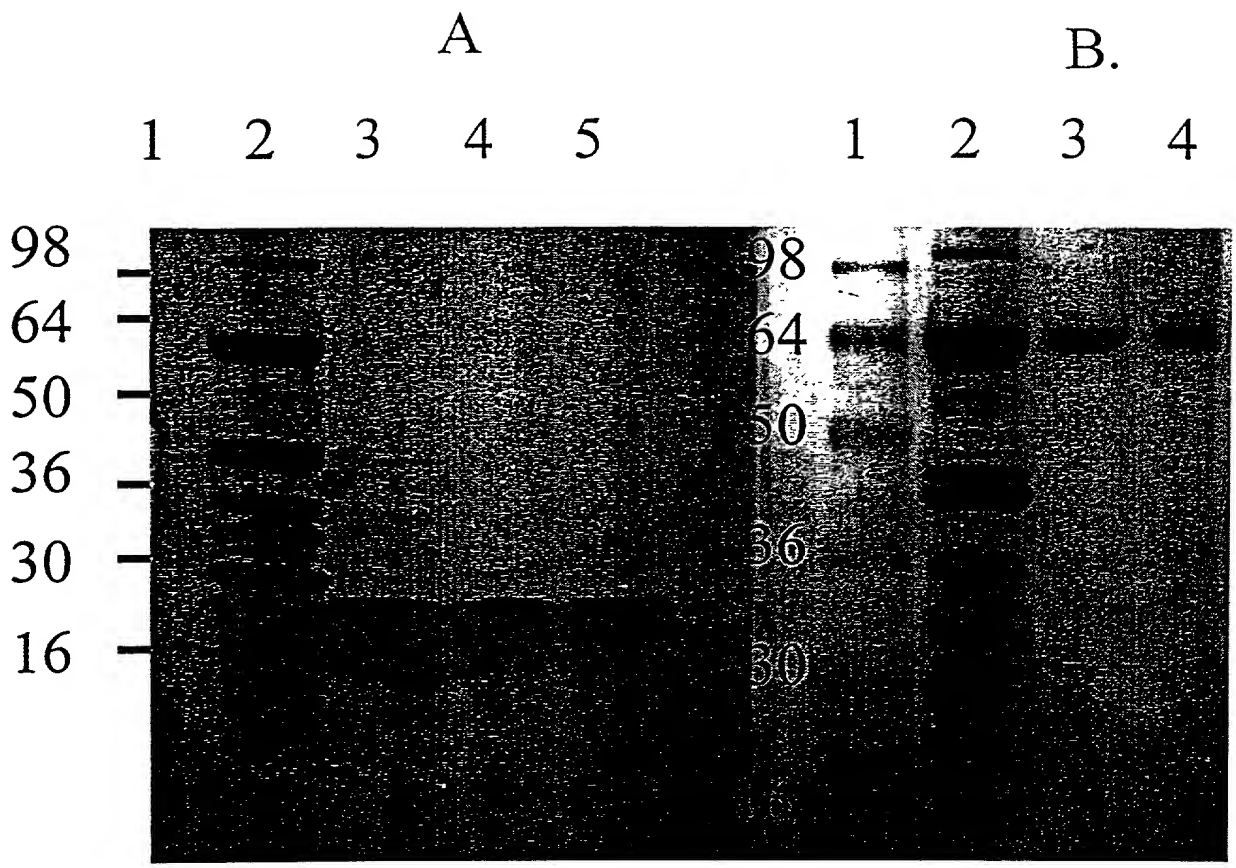


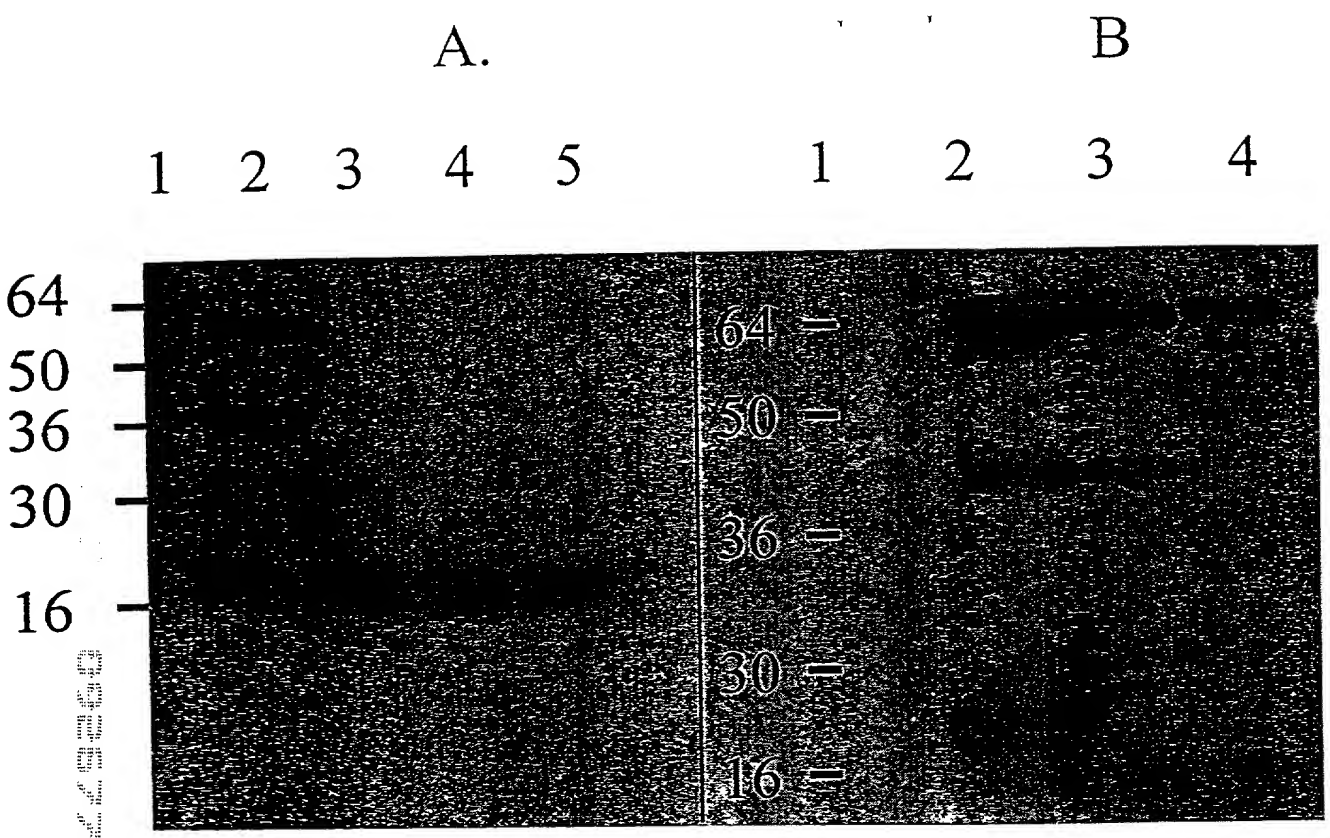
Fig. 16



SDS-PAGE analysis of the protein profiles at various stages of allergen purification.

Panels A and B are the protein profiles resulting from the purification of Ara h 1 and Ara h 2, respectively. Lanes are as follows: *lanes A1 and B1*, protein standards; *lanes A2 and* B2, crude peanut extract; lane *A3*, 25% ammonium sulfate pellet; *A4*, Ara h 2 fraction following anion exchange chromatography; *lane A5*, Ara h 2 fraction following hydrophobic chromatography *lane B3*, 100% ammonium sulfate pellet; *lane B4*, Ara h 1 fraction following cation exchange chromatography.

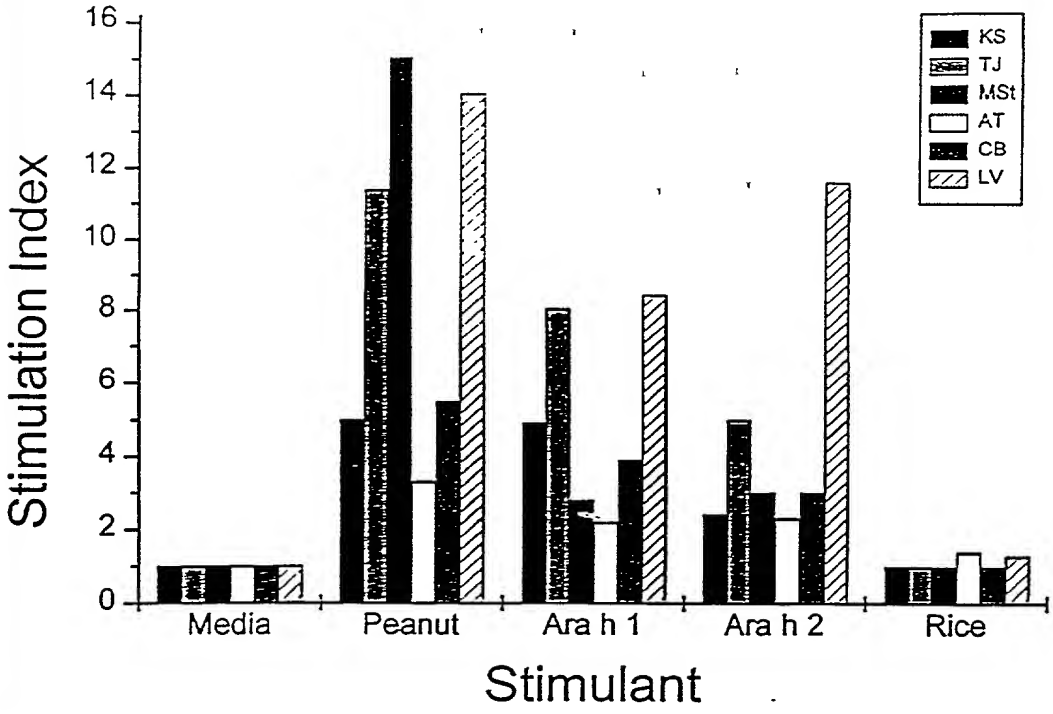
Fig. 17



Immunoblot of the purified proteins using serum IgE from allergic individuals.

Panels A and B are the protein profiles resulting from the purification of Ara h 1 and Ara h 2 that were blotted to nitrocellulose and detected by western blot analysis using serum IgE from allergic individuals as the primary antibody. Lanes are as *follows: lanes A1 and B1*, protein standards; *lanes A2 and* B2, crude peanut extract; lane *A3*, 25% ammonium sulfate pellet; *A4*, Ara h 2 fraction following anion exchange chromatography; *lane A5*, Ara h 2 fraction following hydrophobic chromatography *lane B3*, 100% ammonium sulfate pellet; *lane B4*, Ara h 1 fraction following cation exchange chromatography.

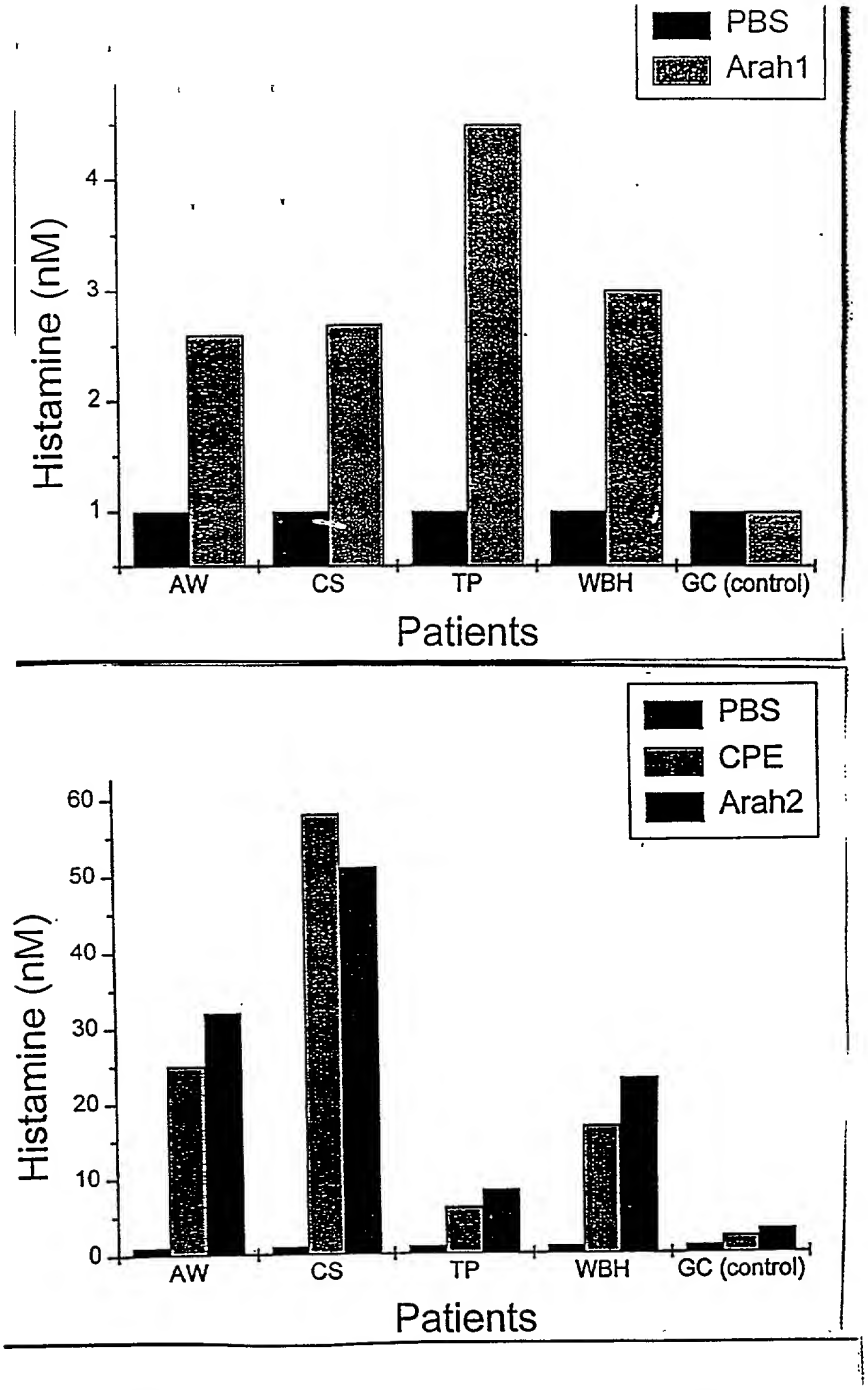
Fig. 18



Purified Ara h 1 and Ara h 2 can stimulate T cell proliferation.

T cells were isolated from peanut allergic individuals and placed into 96 well plates at 4 x 10^4 cells/well and treated in triplicates with media, crude peanut extracts (positive control), Ara h 1, Ara h 2 or rice extracts (negative control). The cells were allowed to proliferate for 6 days and then incubated with ^{3}H-thymidine (1μCi/well) at 37 C for 6-8 hrs and then harvested onto glass fiber filters. T-cell proliferation was estimated by quantitating the amount of ^{3}H-thymidine incorporation into the DNA of proliferating cells. ^{3}H-thymidine incorporation is reported as stimulation (SI) above media treated control cells.

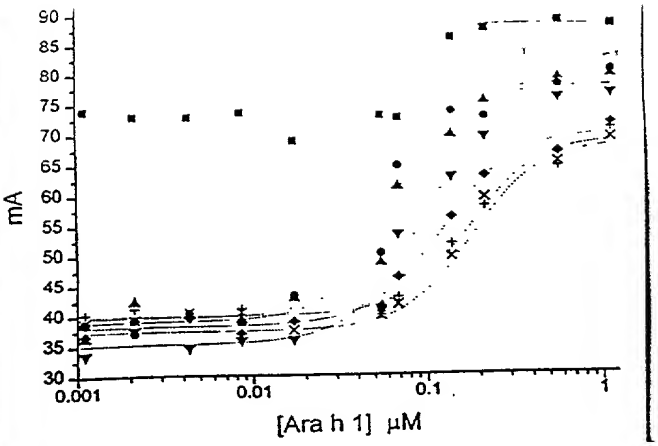
Fig. 19



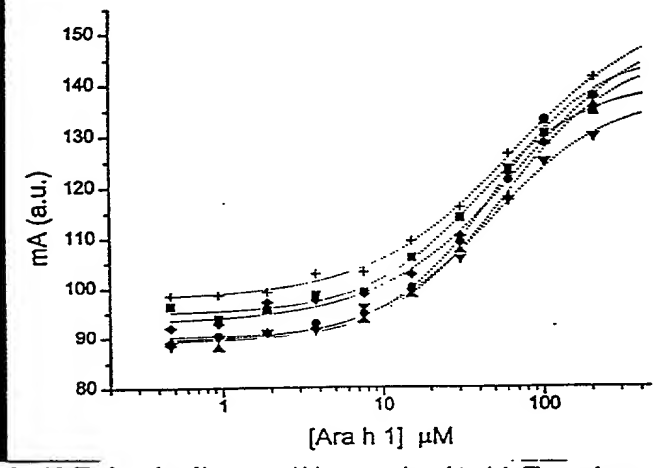
Purified Ara h 1 and Ara h 2 can stimulate histamine release from mast cells of peanut allergic individuals.

Mast cells from whole blood of allergic individuals (on the x-axis) from left to right were treated with PBS (negative control), crude peanut extracts (positive control), Ara h 1 (panel A) or Ara h 2 (panel B). The release of histamine is reported on the y-axis in nM. The histamine release assay was that developed by Immunologics (City, State) and it was performed exactly as described by the manufacturer.

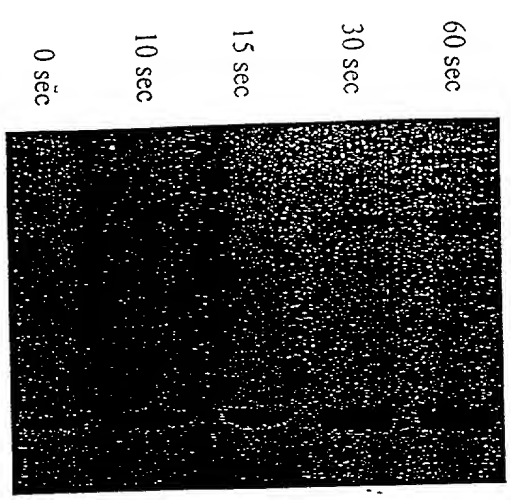
Fig. 20



Panel A: The formation of trimers at low concentrations of Ara h 1. The samples were in binding buffer plus various concentrations of NaCl as indicated: ◯ 0 mM NaCl, ◯ 100 mM NaCl, (·) 300 mM NaCl, (–) 500 mM NaCl (–) 900 mM NaCl, (◫) 1400 mM NaCl, (▽) 1800 mM NaCl.



Panel B: The formation of hexamers at high concentrations of Ara h 1. The samples were in binding buffer plus various concentrations of NaCl as indicated: ◯ 100 mM NaCl, ◯ 400 mM NaCl, (·) 600 mM NaCl, (–) 800 mM NaCl, (–) 1100 mM NaCl, (◫) 1300 mM NaCl, (▽) 1800 mM NaCl.

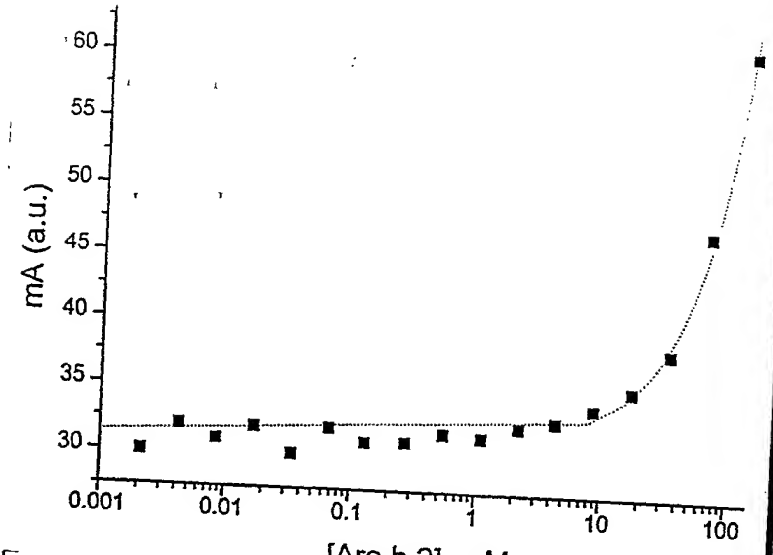


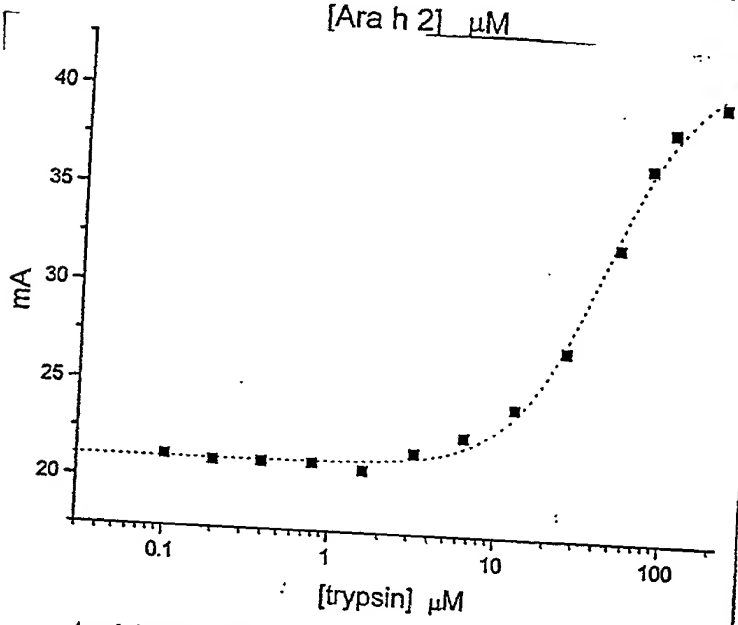
Panel C: Samples from the 80 µM concentration from Panel A were subjected to limited cross-linking at various time points and the products electrophoresed on SDS polyacrylamide gels. Protein bands were visualized by Coomassie staining. Lower arrow indicates the Ara h 1 monomer (~60 kDa), the next highest band represents the Ara h 1 trimer (~180 kDa), and the highest molecular weight band represents Ara h 1 hexamer (~360 kDa)

The purified Ara h 1 protein retains its native structure as indicated by its ability to form homotrimers and hexamers.

Fluorescence anisotropy was used to follow the formation of Ara h 1 higher order structure. All fluorescence measurements were made using a Beacon fluorescence polarization spectrometer (Pan Vera, Madison, WI) with fixed excitation (490 nm) and emission (530 nm) wavelengths which are specified for fluorescein use. Fluorescence measurements were done at room temperature (24 ^{o}C) in binding buffer (50 mM Tris, 1mM EDTA, 100mM NaCl, 2mM DTT, 5% glycerol, pH 7.5) in a final volume of 1.1 ml. A constant amount of fluorescein labeled protein (10 nM of Ara h 1) was diluted with binding buffer and mixed with various concentrations of unlabeled Ara h 1 to analyze homo-oligomer formation. Serial dilutions of the desalted proteins (by 0.5 or 0.8 increments) were made in binding buffer and the appropriate amounts were added to constant amounts of fluorescein-labeled protein. Each data point is an average of three independent measurements. The intensity of fluorescence remained constant throughout the anisotropy measurements.

Fig. 21





Ara h 2 does not form homo-oligomers but does bind to trypsin.

The fluorescence milli-anisotropy (mA) of fluorescent-labeled Ara h 2 titrated with unlabeled Ara h 2 or trypsin at different concentrations is measured and plotted. All fluorescent measurements were performed exactly as described in Fig. 5.

Panel A: The fluorescence milli-anisotropy (mA) of fluorescent-labeled Ara h 2 titrated with unlabeled Ara h 2 at different concentrations is measured and plotted versus the concentration of unlabeled Ara h 2. The samples were in binding buffer plus ( )100 mM NaCl

Panel B: The fluorescence milli-anisotropy (mA) of fluorescent-labeled Ara h 2 titrated with unlabeled trypsin at different concentrations is measured and plotted versus the concentration of unlabeled trypsin. The samples were in binding buffer plus ( ) 100 mM NaCl.

Fig. 22

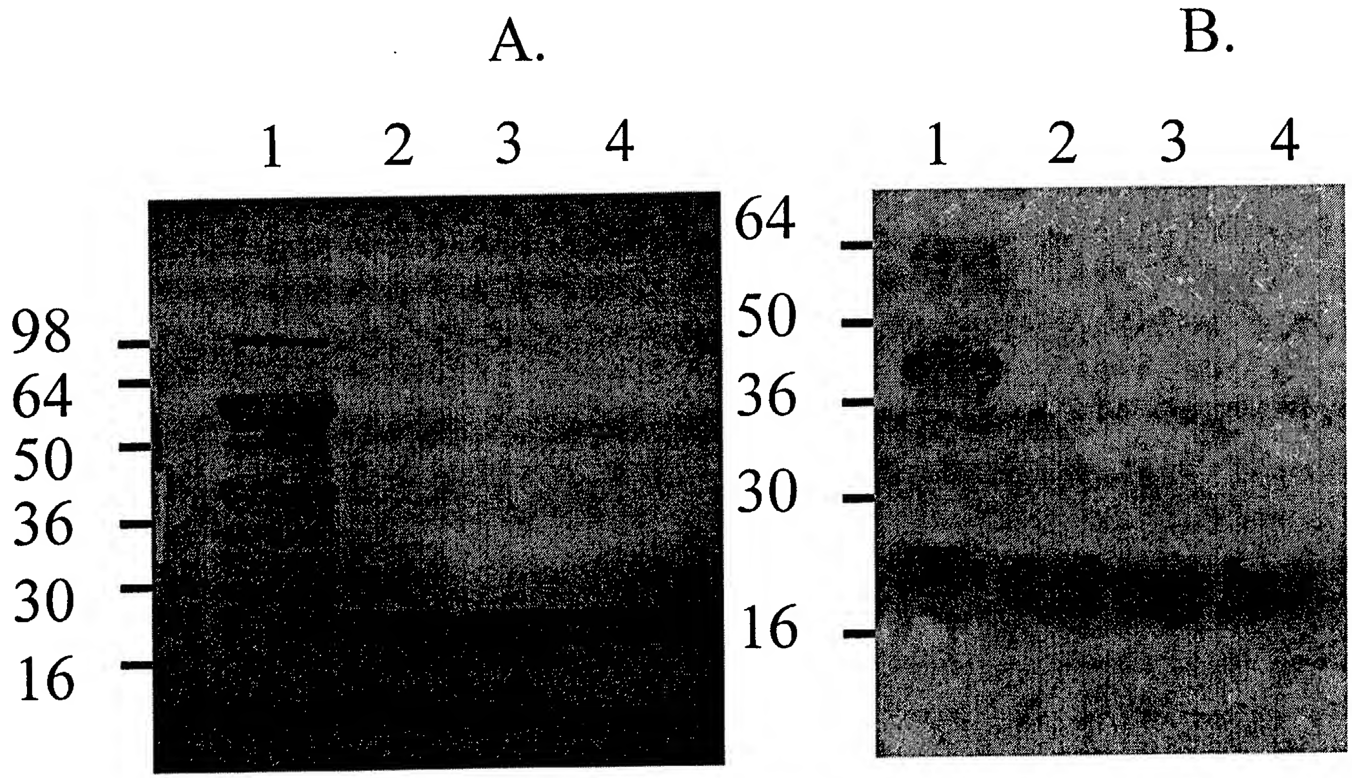


Fig.23

Anti-Zap-70 Ip, Anti-TCR western

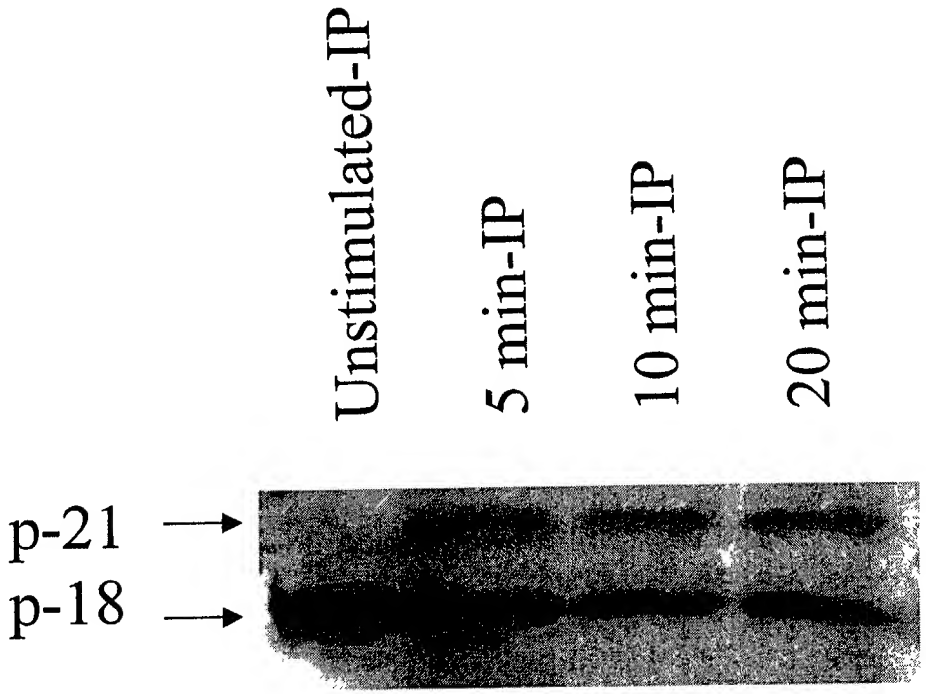


Fig. 24A

Anti-Zap-70-IP, Anti-pTyr western.

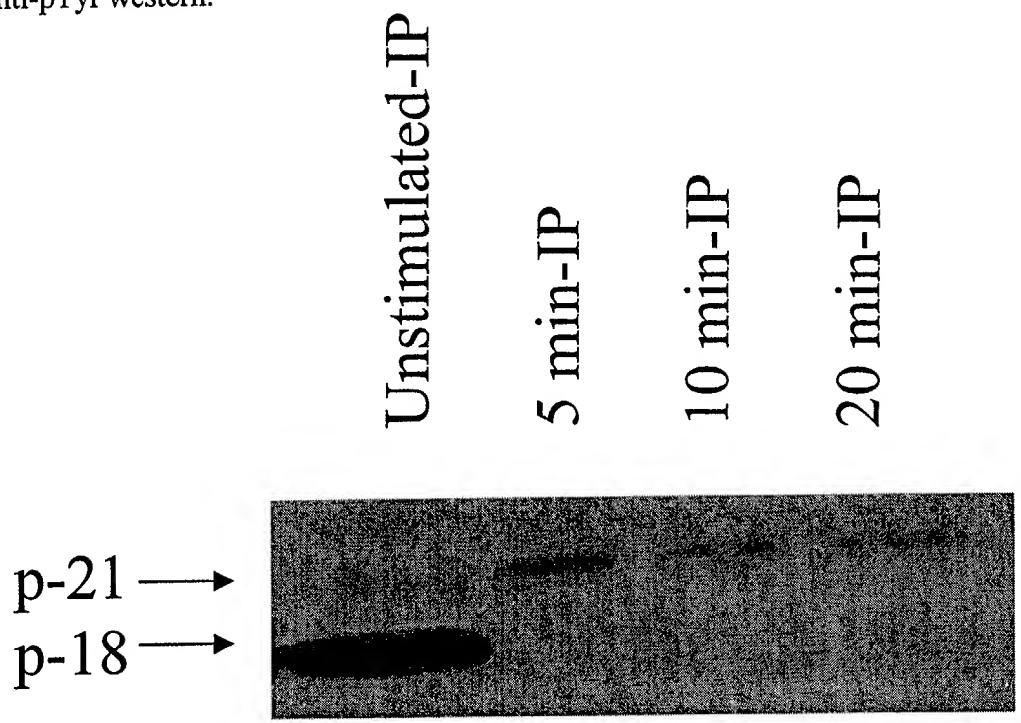


Fig. 24B

Anti-TCR-IP, Anti-ERK-1 western

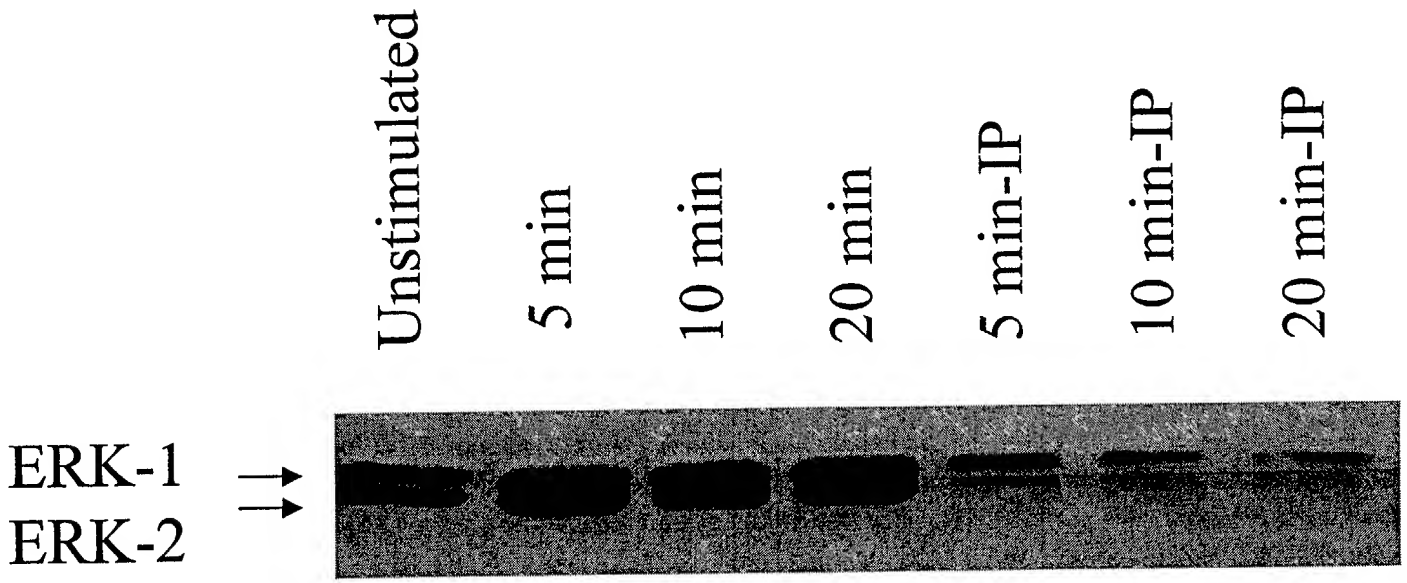


Fig. 24C

Anti-TCR-IP, Anti pTYR western.

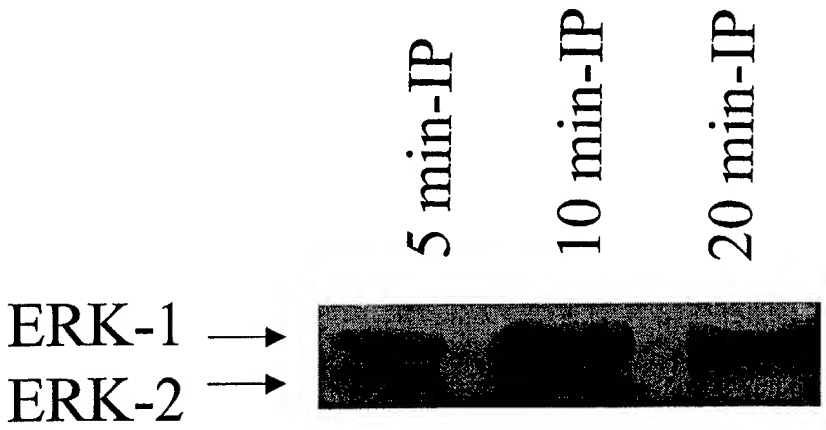


Fig. 24D

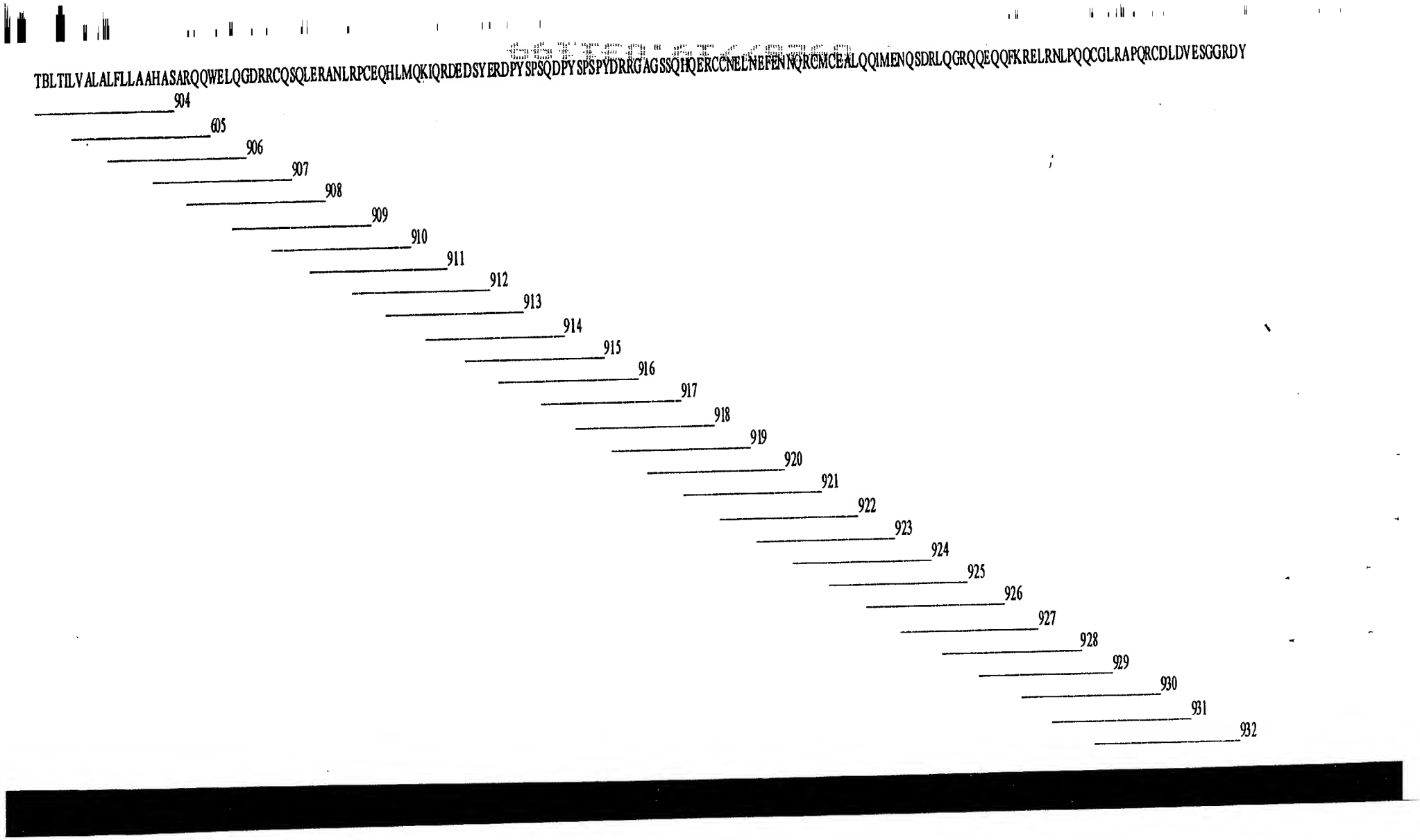


Fig.25 Synthetic overlapping peptides of Ara h 2. In order to determine the T-cell epitopes of peanut allergen Ara h 2, 29 different peptides representing the entire protein were synthesized. Each peptide was 20 amino acids long and was offset from the previous peptide by 5 amino acids. In this manner we were able to cover the entire protein sequence by overlapping peptides. The primary amino acid sequence of the Ara

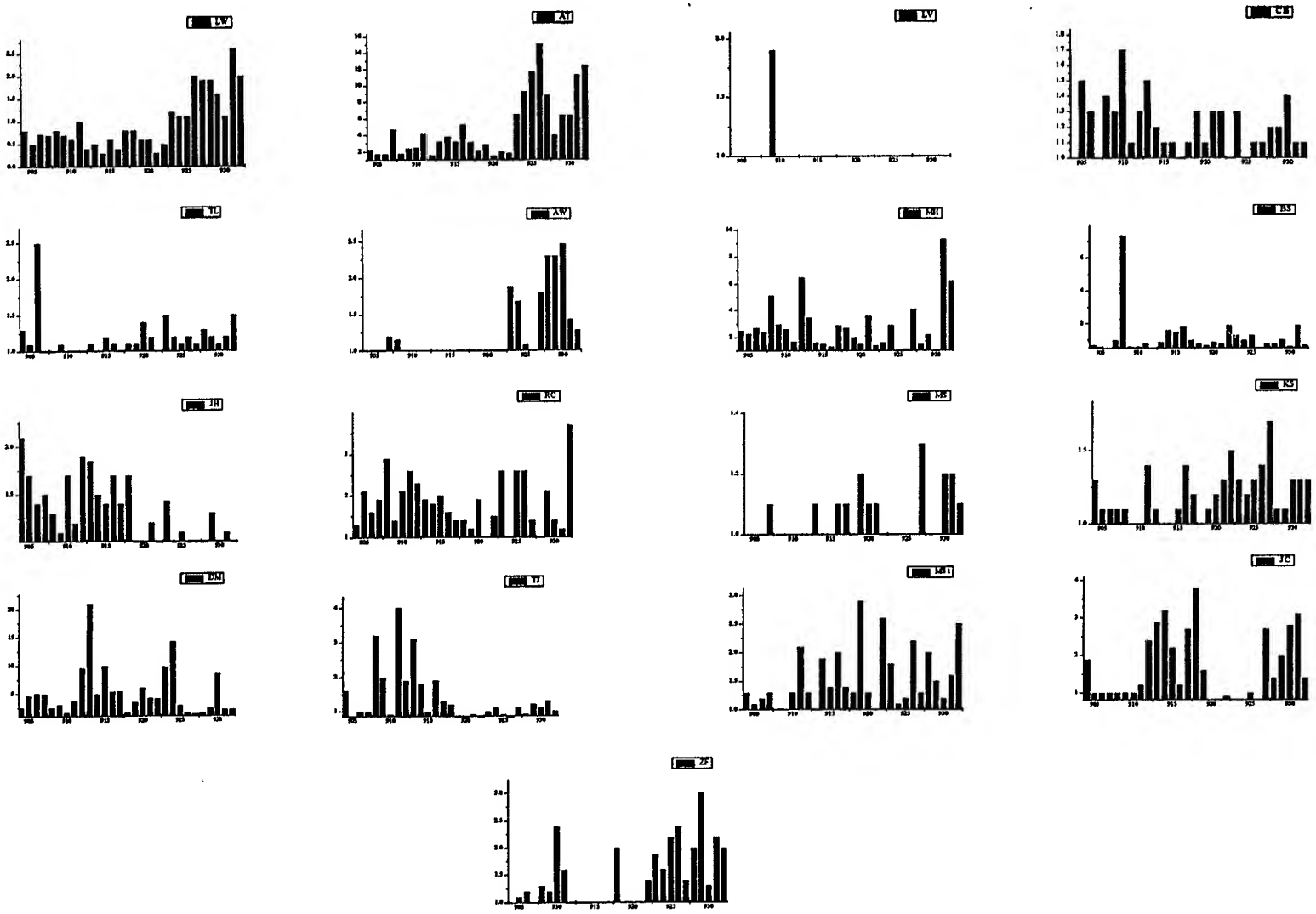
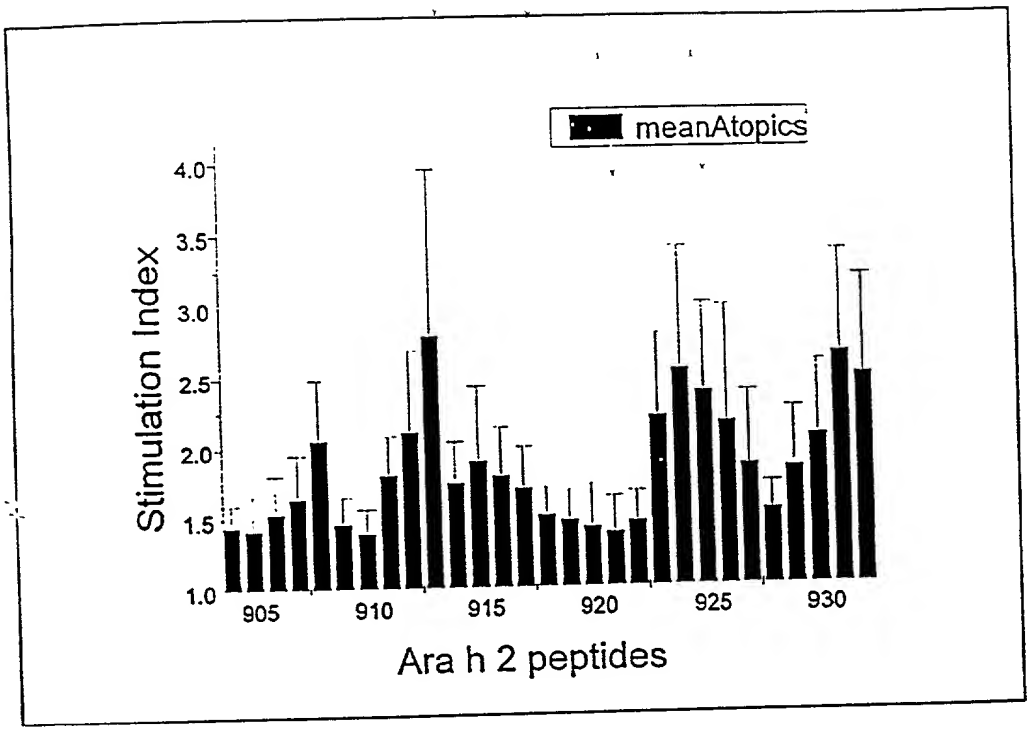
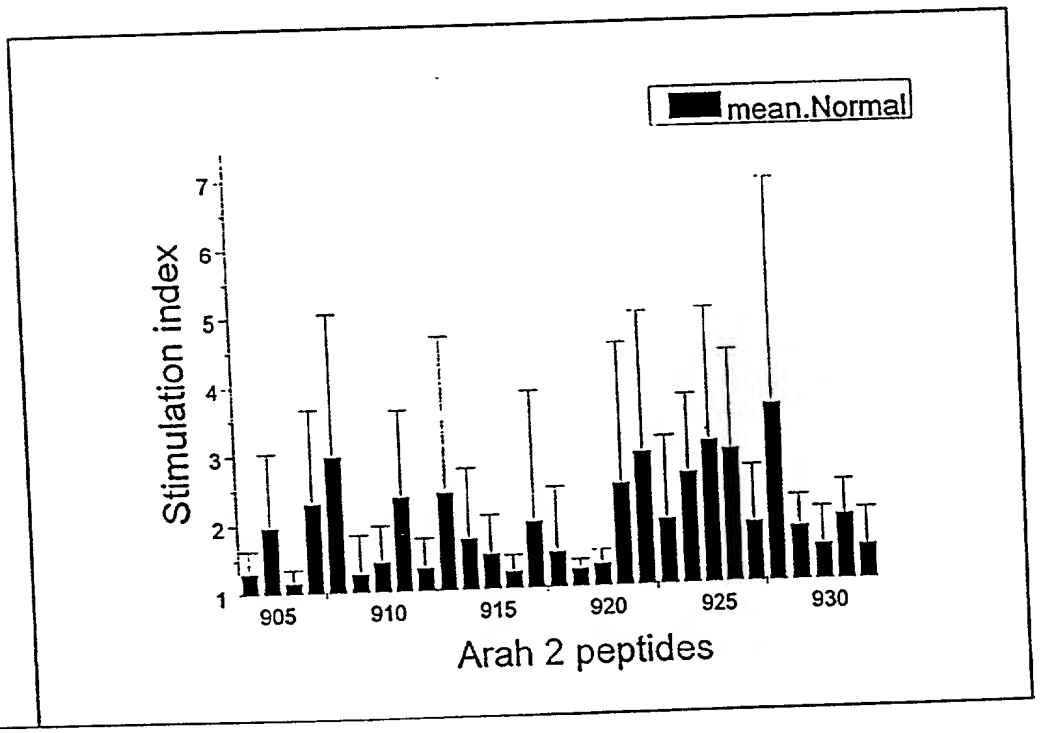


Fig. 26

Panel A



Panel B

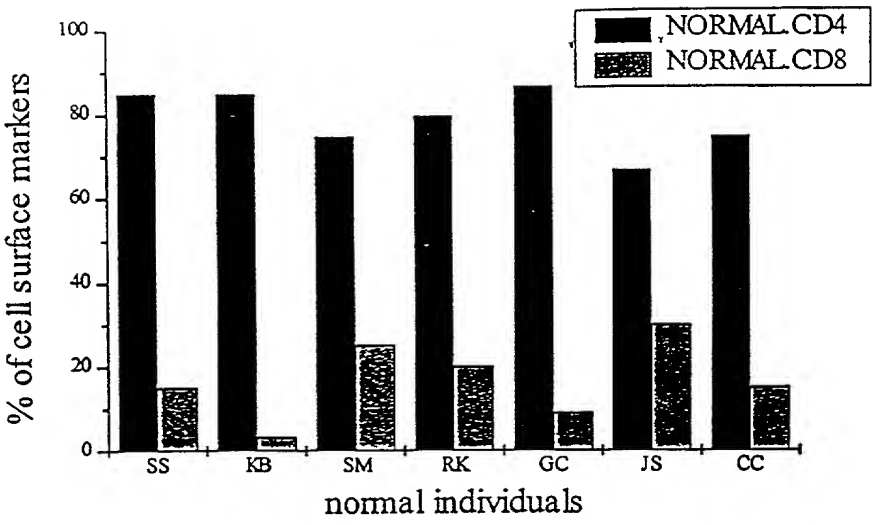


Identification of the Ara h 2 peptides that caused T-cell proliferation in the majority of patients tested.

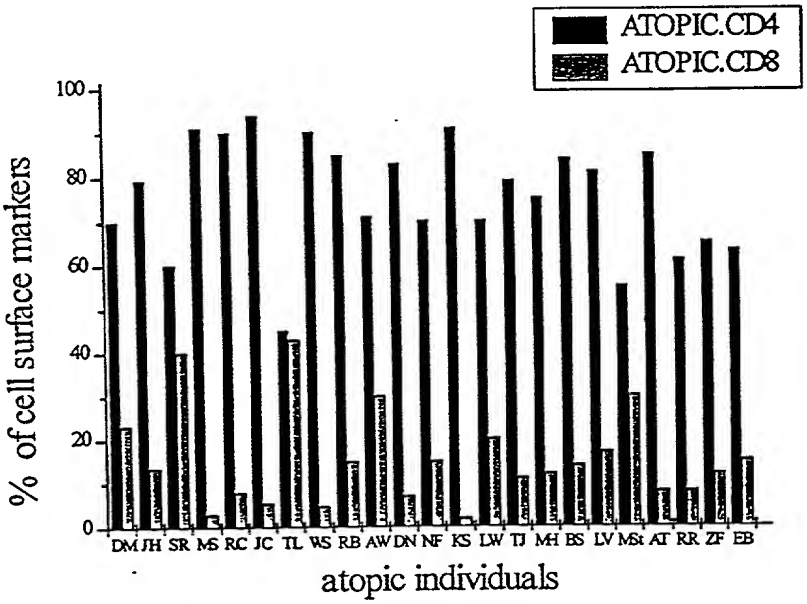
All of the data in Figure 2 was compiled and plotted as a stimulation index versus the Ara h 2 peptides. The mean proliferation and standard error of (panel A) 17 peanut allergic individuals and (panel B) 5 non-allergic individuals were calculated and plotted as mean stimulation index of atopic individuals versus the 29 overlapping peptides spanning the entire Ara h 2 protein from the amino-(peptide 904) to carboxyl terminus (peptide 932).

Fig. 27

Panel A



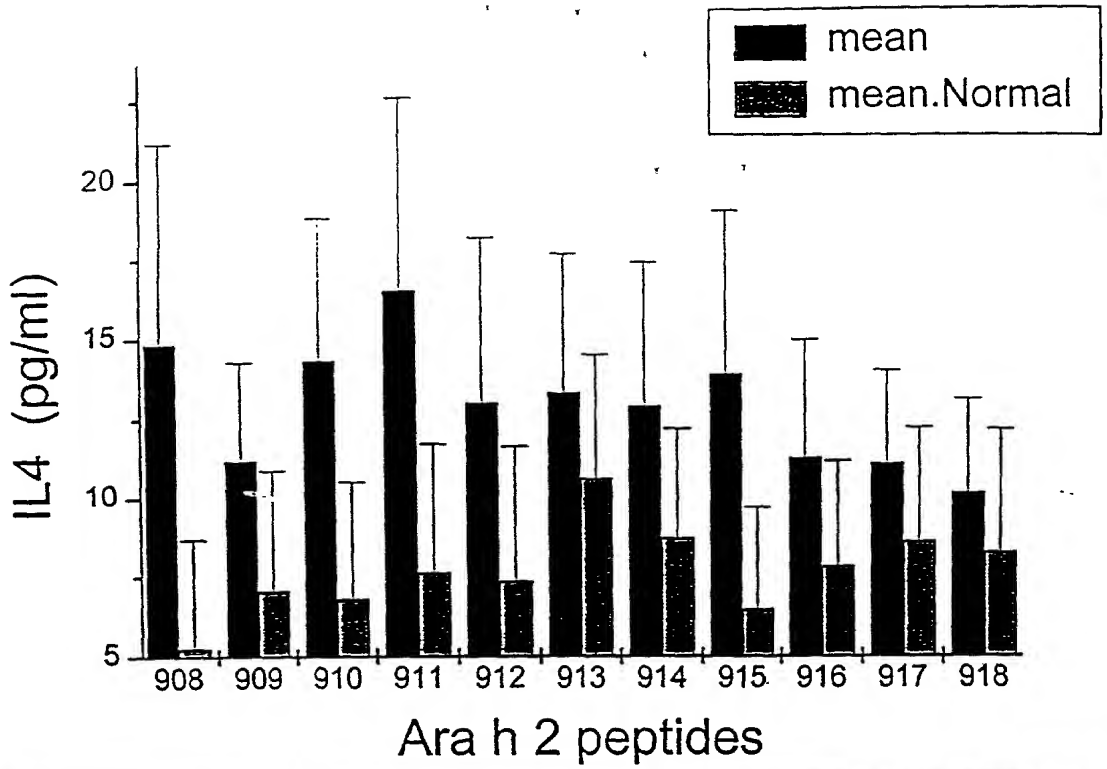
Panel B



The CD4$^+$ and CD8$^+$ profiles of the T-cell lines of peanut allergic individuals.

T cells were stained with FITC-labeled anti-CD4 and FITC-labeled anti-CD8 antibodies in order to determine the phenotype of the peanut specific T-cell lines established. FACS analysis was used to determine the percent of CD4$^+$ and CD8$^+$ cells in the peanut specific T-cell lines utilized in Ara h 2 epitope mapping and plotted versus the initials of the individual patients used to establish these cell lines. Panel A represents the CD4/CD8 profiles of T-cell lines established from allergic individuals while panel B represents the CD4/CD8 profiles of T-cell lines established from non-allergic individuals.

Fig. 28



The IL-4 secretion profiles of a representative sample of T cells.

The supernatant was collected from T-cells stimulated with immunodominant peptides and an ELISA assay was utilized to measure IL-4 concentrations in the media. IL-4 concentration is plotted versus the 29 overlapping peptides spanning the entire Ara h 2 protein from amino- (peptide 904) to carboxyl terminus (peptide 932).

Fig. 29

T-1

TILVALALFLLAAHASARQQWELQGDRRCQSQLERANRP

B-3

T-2

CEQHLMQKIQRDEDSYERDPYSPSQDPYSPSPYDRRAGS

B-6 B-7

T-3

SQHQERCCNELNEFENNQRCMCEALQQIMENQSRLQGRQ

T-4

QEQQFKRELRNLPQQCGLRAPQRCDLDVESGRDY

B-CELL EPITOPES

T-CELL EPITOPES

Comparison of the T-cell and B-cell epitopes of Ara h 2.

The primary amino acid sequence of the Ara h 2 protein is represented as the one letter amino acid code. The T-cell epitopes of Ara h 2 that have been identified in this study are depicted as bold, italicized letters and the immunodominant B-cell epitopes determined in previous work are underlined. In general, the IgE binding epitopes do not overlap with the T-cell epitopes.

Fig. 30

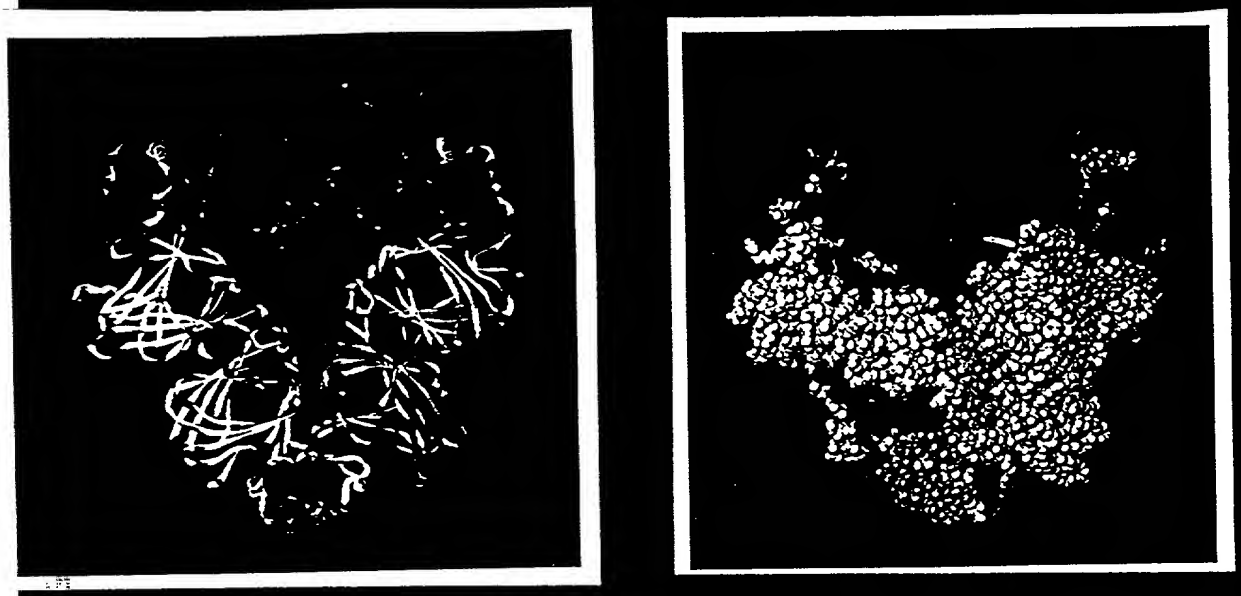


Fig. 3| Trimeric model of the Ara h 1 molecule.

It has been shown that other vicilin homologs form trimeric structures. Ara h 1 was also determined to form a trimeric structure by fluorescence anisotropy and cross linking experiments (see poster # 994). Ara h 1 was modeled in trimeric form by aligning the constructed model (see fig. 1 and 4) to monomers A, B and C of the trimeric template molecule phaseolin. The left panel is a ribbon diagram of the trimer with each monomer represented in a different color. The right panel is a space filled diagram where the epitope regions on the white monomer are shown in red and the epitope regions on the yellow monomer are shown in orange. This shows that a clustered epitope region on one side of a monomer joins the opposite clustered epitope region of another.

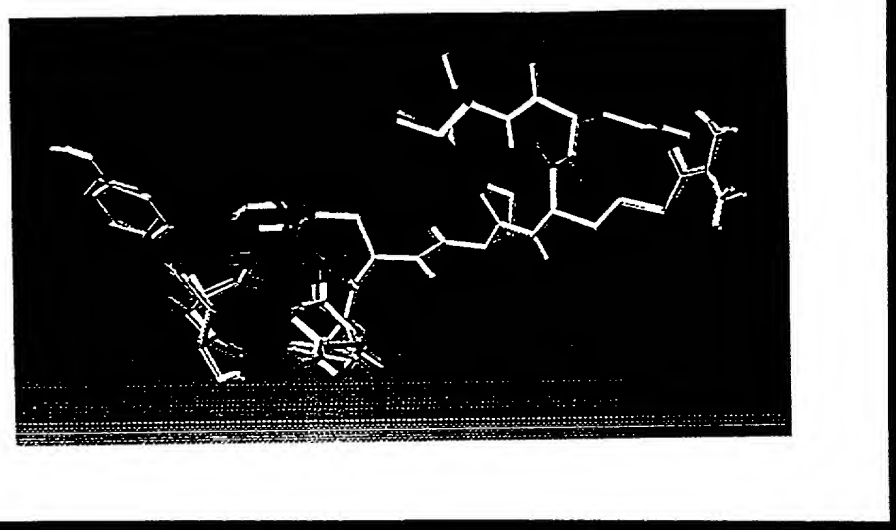


Fig. 32 Determination of residues targeted for mutagenesis in the Ara h 1 gene.

Residues that were found to abolish IgE binding through peptide mutagenesis (see Fig. 2 and Table 1) were mutated and analyzed in the molecular model to establish which residues would less likely interfere with protein stability. The wild type form of epitope 11, SYLQEFSRNT, is shown in white with the leucine chosen to be mutated in red. The gold peptide is the mutated form with a methionine substitution shown in purple. The mutant was locally annealed. This substitution is predicted to leave the molecule in the least disturbed conformation and has the lowest energy compared to the other residues that were shown to abolish IgE binding when mutated (shown in bold: SYLQEFSRNT). The other modeled epitopes were analyzed using the same method and one or more choices per epitope appeared to be suitable for mutagenesis without altering the tertiary structure of Ara h 1 significantly.

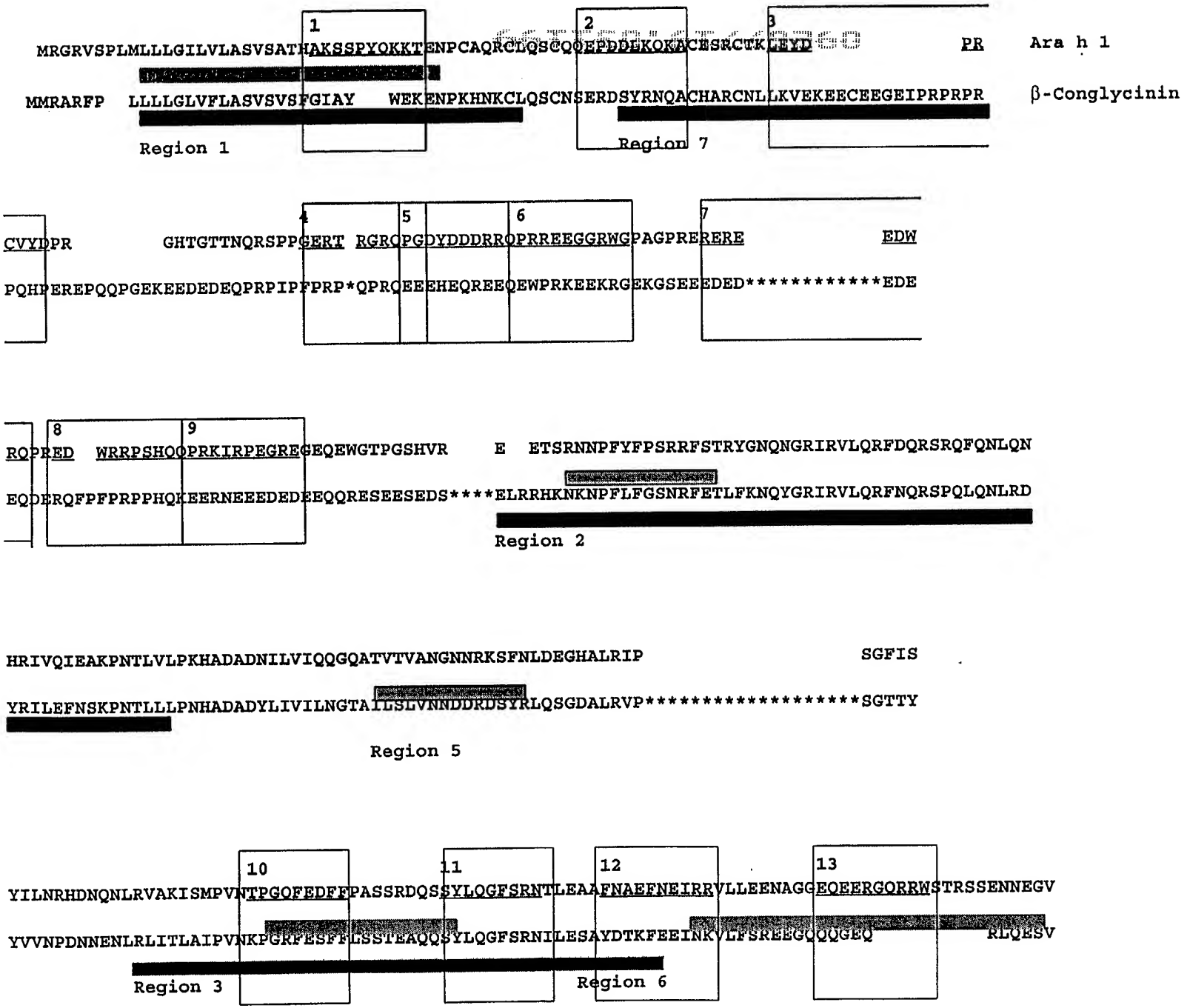
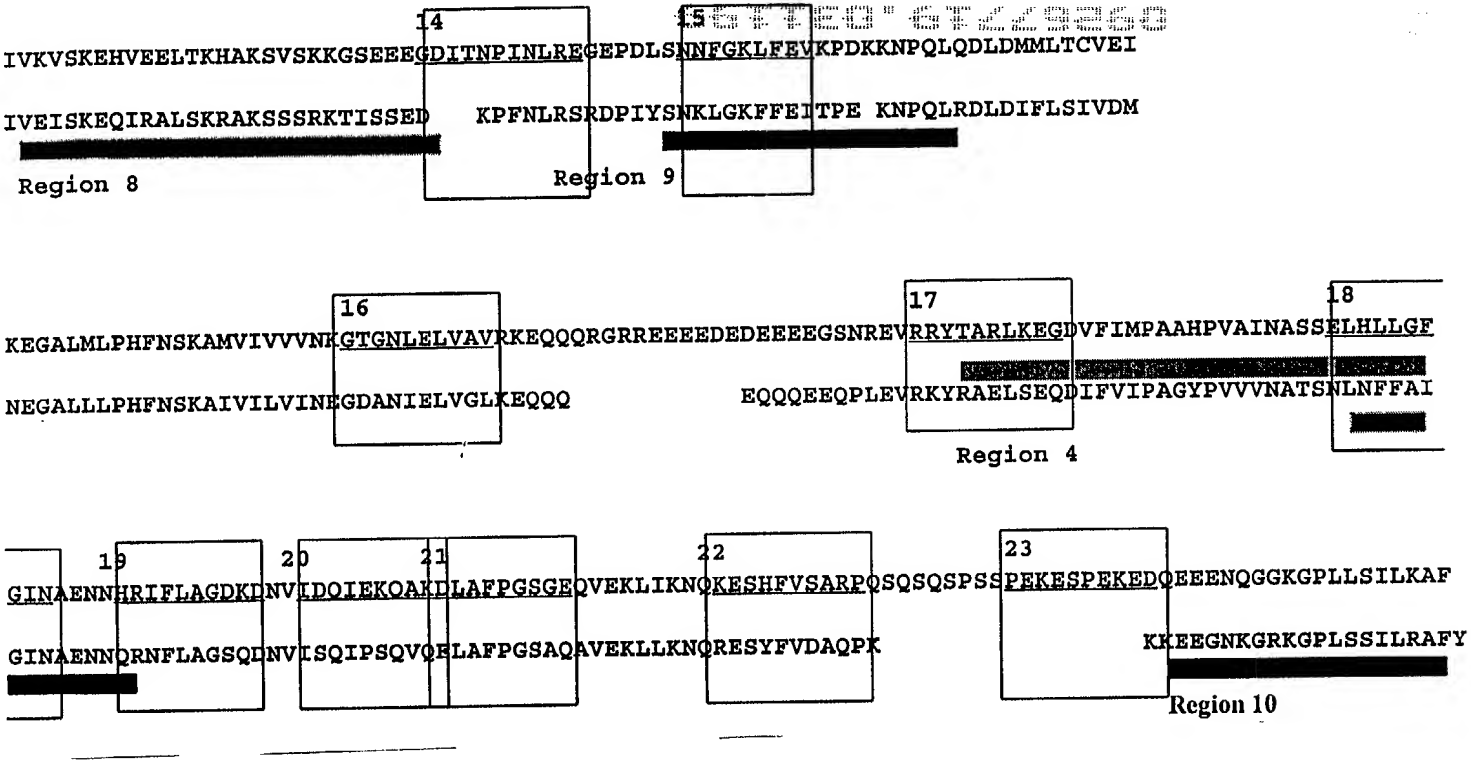

1
2
3
MRGRVSPLMLLLGILVLASVSATHAKSSPYQKKTENPCAQRCLQSCQQEPDDLKQKACESRCTKLEYD PR
Ara h 1
MMRARFP LLLLGLVFLASVSVSFGIAY WEKENPKHNKCLQSCNSERDSYRNQACHARCNLLKVEKEECEEGEIPRPRPR
β-Conglycinin
Region 1
Region 7
4
5
6
7
CVYDPR GHTGTTNQRSPPGERT RGRQPGDYDDDRRQPRREEGGRWGPAGPRERERE EDW
PQHPEREPQQPGEKEEDEDEQPRPIPFPRP*QPRQEEEHEQREEQEWPRKEEKRGEKGSEEEDED**********EDE
8
9
RQPRED WRRPSHQQPRKIRPEGREGEQEWGTPGSHVR E ETSRNNPFYFPSRRFSTRYGNQNGRIRVLQRFDQRSRQFQNLQN
EQDERQFPFPRPPHQKEERNEEEDEDEEQQRESEESEDS****ELRRHKNKNPFLFGSNRFETLFKNQYGRIRVLQRFNQRSPQLQNLRD
Region 2
HRIVQIEAKPNTLVLPKHADADNILVIQQGQATVTVANGNNRKSFNLDEGHALRIP SGFIS
YRILEFNSKPNTLLLPNHADADYLIVILNGTAILSLVNNDDRDSYRLQSGDALRVP**********SGTTY
Region 5
10
11
12
13
YILNRHDNQNLRVAKISMPVNTPGQFEDFFPASSRDQSSYLQGFSRNTLEAAFNAEFNEIRRVLLEENAGGEQEERGQRRWSTRSSENNEGV
YVVNPDNNENLRLITLAIPVNKPGRFESFFLSSTEAQQSYLQGFSRNILESAYDTKFEEINKVLFSREEGQQQGEQ RLQESV
Region 3
Region 6

Fig. 33A



SOYBEAN SEQUENCE HOMOLOGY TO ARA H 1
SOYBEAN AND PEANUT POSITIVE REGIONS
15-MERS X 8 SPOTS ANALYSIS

Soybean IgE Positive Binding Regions

Ara h 1 Epitopes

Peanut IgE Positive Binding Regions

Fig. 33B

SEQUENCE HOMOLOGY OF ARA H I IgE EPITOPES IN REGIONS OF BETA CONGLYCININ

```
EPITOPE 1
AKSSPYQKKT
GIAY   WEK

EPITOPE 2
QEPDDLKQKA
SERDSYRNQA
 * *    *

EPITOPE 3
LEYD              PRLVYD
LKVEKEECEEGEIPRPRPRPQHP
*                 **

EPITOPE 4
GERTRGRQPG
FPRPQPRQEE
  *   **

EPITOPE 5
PSDYDDDRRQ
EEEHEQREEQ
          *

EPITOPE 6
PRREEGGRWG
EWPRKEEKRG
         *

EPITOPE 7
REREEDWRQP
EDEDEDEDEQ
      *

EPITOPE 8
EDW  RRPSHQQ
RQFPFPRPPHQK
     **  **

EPITOPE 9
QPRKIRPEGR
KEERNEEEDE
       *

EPITOPE 10
TPGQFEDFFP
KPGRFESFFL
 ** ** **

EPITOPE 11
SYLQEFSRNT
SYLQGFSRNI
**** ****

EPITOPE 12
FNAEFNEIRR
YDTKEEINKV

EPITOPE 13
EQEERGQRRW
QQGEQRILQE
 * *

EPITIPE 14
DITNPINLRE
   KPFNLRS
  * ***

EPITOPE 15
NNFGKLFEVK
NKLGKFFEIT
*  ** **

EPITOPE 16
GTGNLELVAV
GDANIELVGL
*  * ***

EPITOPE 17
RRYTARLKEG
RKYREELSEQ
* *   *

EPITOPE 18
ELHLLGFGIN
NLNFFAIGIN
 *     ***

EPITOPE 19
JRIFLAGDKD
QRNFLAGSQD
   ****  *

EPITOPE 20
IDQIEKQAKD
ISQIPSQVQE
* **   *

EPITOPE 21
KDLAFPGSGE
QELAFPGSAQ
  ******

EPITOPE 22
KESKFVSARP
RESYFVDAQP
 ** ** * *

EPITOPE 23
PEKESPEKED
         K
```

Fig. 34

cDNA CLONING

Soybean seeds, Glycinus max, Hutchinson variety, were obtained from a local health food store, frozen in liquid nitrogen, ground to a fine powder, and the RNA extracted using the method of Nedergaard et al (Mol Immunol 29:703,1992). Briefly, 2 g frozen seed powder was added to 10 mls buffer (250 mM sucrose, 200 mM Tris-HCl, pH 8.0, 200 mM KCl, 30 mM $MgCl_2$, 2% polyvinylpyrrokidone-40 and 5 mM 2-mercaptoethanol) and equilibrated with 10 ml fresh phenol (4°C). The suspension was homogenized and 10 ml of chloroform added with shaking for 5 min at RT. Phases were separated by centrifugation, 10k g for 20 min at 4°C and the aqueous phase transferred to a clean test tube and extracted 2x with equal volumes of chloroform/phenol. Nucleic acids were precipitated with sodium acetate/ethanol at -20°C overnight. The precipitates were collected by centrifugation at 13k g for 20 min at 4°C, washed with 70% ethanol and dried. Samples run in parallel were pooled in water and made 3M in LiCl, and the RNA precipitated for 4 hr at -20°C. The precipitate was collected by centrifugation outlined above and resuspended in distilled water. Fifty microliters of the RNA suspension was withdrawn for OD260/280 measurements and the RNA analyzed by agarose gel electrophoresis. Three aliquots representing a total of approximately 3.0 mgs total RNA was sent to STRATAGENE for purification of mRNA and the preparation of a Uni-Zap XR custom library.

The expression custom library was screened with serum from soybean-sensitive individuals and positive clones subcloned to homogeneity with respect to IgE-binding. Five clones were isolated from an initial screen and the plasmids purified from LB/ampicilin broth cultures using an Ameresco kit. The plasmid DNA from each clone was PCR amplified and analyzed in agarose gels. Two plasmid preparations had relative bp of approximately 1400 and the remaining three 1500 bp.

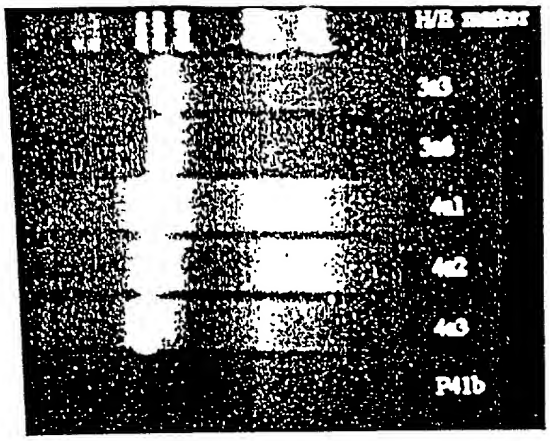


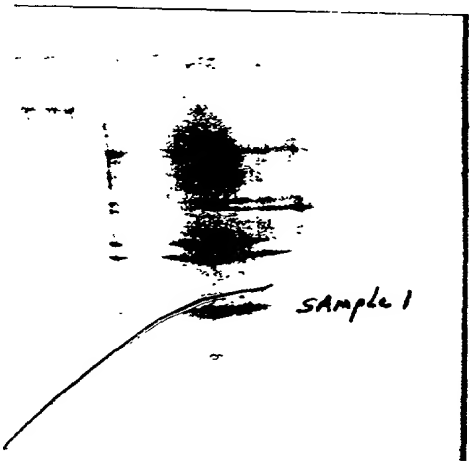
Fig. 35

PCR AMPLIFIED PLASMIDS ISOLATED FROM SOYBEAN CDNA EXPRESSION LIBRARY

ALLERGEN IDENTIFICATION BY 2-D SDS-PAGE

A crude soybean extract was applied to a 12.5% preparative SDS-PAGE gel and electrophoresed using a BIO-RAD prep cell. Five ml fractions were collected and aliquots were electrophoresed into a Pharmacia 24-well 10% horizontal gel, electrophoretically transferred to a nitrocellulose membrane, the remaining sites blocked using PBS/0.05% Tween 20, and analyzed for IgE-binding using serum from soybean-sensitive individuals. Fractions that bound IgE were dialyzed against 100mM ammonium bicarbonate (x4 x 4 liters) for 24 hours, lyophilized, reconstituted in distilled water and analyzed by 2-D (isoelectric focusing in the first dimension, pH 3-7, followed by a 4-20% SDS-PAGE gel molecular weight separation in the second) in duplicate. The proteins in the duplicate gels were transferred to nitrocellulose membranes, one was stained with Coomassie blue for protein identification and the other was prepared for IgE immunoblot analysis. IgE-binding proteins were identified by radiolabeled anti-IgE and X-ray autoradiography. Positive IgE-binding proteins by autoradiography were compared to the Coomassie stained gel protein profile. The stained blot was submitted to the Yale Biotechnology Center for amino acid sequencing. The results of this analysis revealed a 20-22kD protein with significant homology to the A2B1a glycinin protein family. Additional samples are being assessed for activity and identification.

A: Coomassie blue stained 2-D SDS-PAGE gel



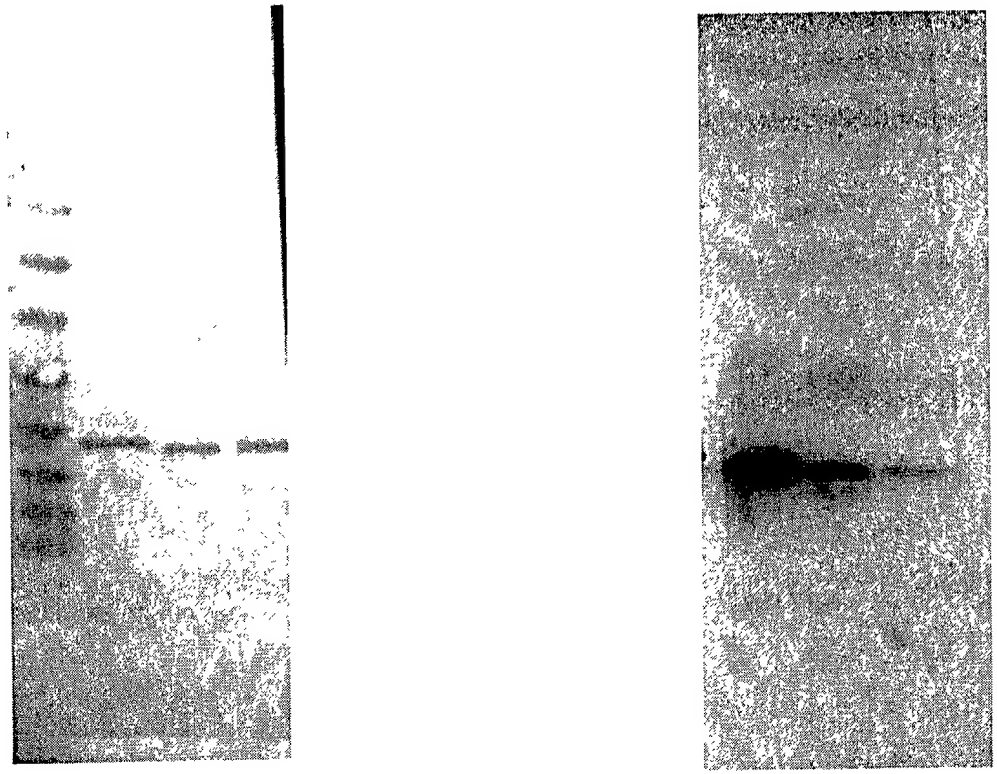
B: IgE immunoblot of 2-D SDS-PAGE blot

Fig. 36

Figure 3A

IgE BINDING OF rAra h 2 PROTEINS IN WESTERN BLOT ANALYSIS

T7 tag **Serum IgE**



MW 10 6 0 **10 6 0**

Number of epitopes

Figure 37 B

SERUM IgE BINDING OF rAra h 2 PROTEINS IN INDIVIDUAL PATIENTS

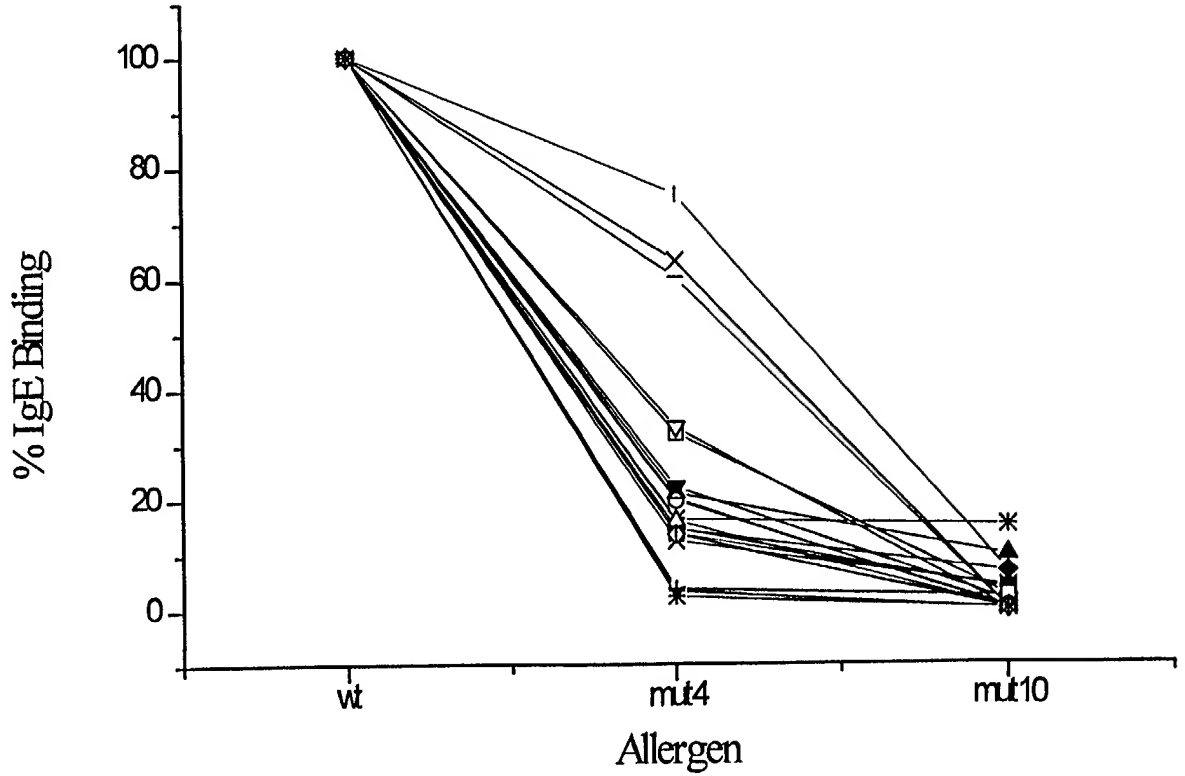


Fig. 38 Upper Panel

Fig. 38 Lower Panel

Fig 39 Upper Panel

Ara h 1- Pepsin/Chymotrypsin Sites

MRGRVSPLMLLLGILVLASVSATHAKSSPYQKKTENPCAQRCLQSCQQEP

DDLKQKACEESRCTKLEYDPRCVYDPRGHTGTTNQRSPPGERTRGRQPGD

YDDDRRQPRREEGGRWGPAGPRERERREEDWRQPREDWRRPSHQQPRKIRP

EGREGEQEWGTPGSHVREETSRNNPFYFPSRRFSTRYGNQNGRIRVLQRFD

QRSRQFQNLQNHRIVQIEAKPNTLVLPKHADADNILVIQQGQATVTVANGN

NRKSFNLDEGHALRIPSGFISYILNRHDNQNLRAAKISMPVNTPGQFEDFFPA

SSRDQSSYLQGFSRNTLEAAFNAEFNEIRRVLLEENAGGEQEERGQRRWST

RSSENNEGVIVKVSKEHVEELTKHAKSVSKKGSEEEGDITNPINLREGEPDLS

NNFGKLFEVKPDKKNPQLQDLDMMLTCVEIKEGALMLPHFNSKAMVIVVV

NKGTGNLELVAVRKEQQQRGRREEEEDEDEEEEGSNREVRRYTARLKEGD

VFIMPAAHPVAINASSELHLLGFGINAENNHRIFLAGDKDNVIDQIEKQAKD

LAFPGSGEQVEKLIKNQKESHFVSARPQSQSQSPSSPEKESPEKEDQEEENQG

GKGPLLSILKAFN

[redacted]

Fig. 39 Lower Panel

Ara h 1- Trypsin Sites

MRGRVSPLMLLLGILVLASVSATHAKSSPYQKKTENPCAQRCLQSCQQEP

DDLKQKACEESRCTKLEYDPRCVYDPRGHTGTTNQRSPPGERTRGRQPGD

YDDDRRQPRREEGGRWGPAGPRERERREEDWRQPREDWRRPSHQQPRKIRP

EGREGEQEWGTPGSHVREETSRNNPFYFPSRRFSTRYGNQNGRIRVLQRFD

QRSRQFQNLQNHRIVQIEAKPNTLVLPKHADADNILVIQQGQATVTVANGN

NRKSFNLDEGHALRIPSGFISYILNRHDNQNLRAAKISMPVNTPGQFEDFFPA

SSRDQSSYLQGFSRNTLEAAFNAEFNEIRRVLLEENAGGEQEERGQRRWST

RSSENNEGVIVKVSKEHVEELTKHAKSVSKKGSEEEGDITNPINLREGEPDLS

NNFGKLFEVKPDKKNPQLQDLDMMLTCVEIKEGALMLPHFNSKAMVIVVV

NKGTGNLELVAVRKEQQQRGRREEEEDEDEEEEGSNREVRRYTARLKEGD

VFIMPAAHPVAINASSELHLLGFGINAENNHRIFLAGDKDNVIDQIEKQAKD

LAFPGSGEQVEKLIKNQKESHFVSARPQSQSQSPSSPEKESPEKEDQEEENQG

GKGPLLSILKAFN

[redacted]

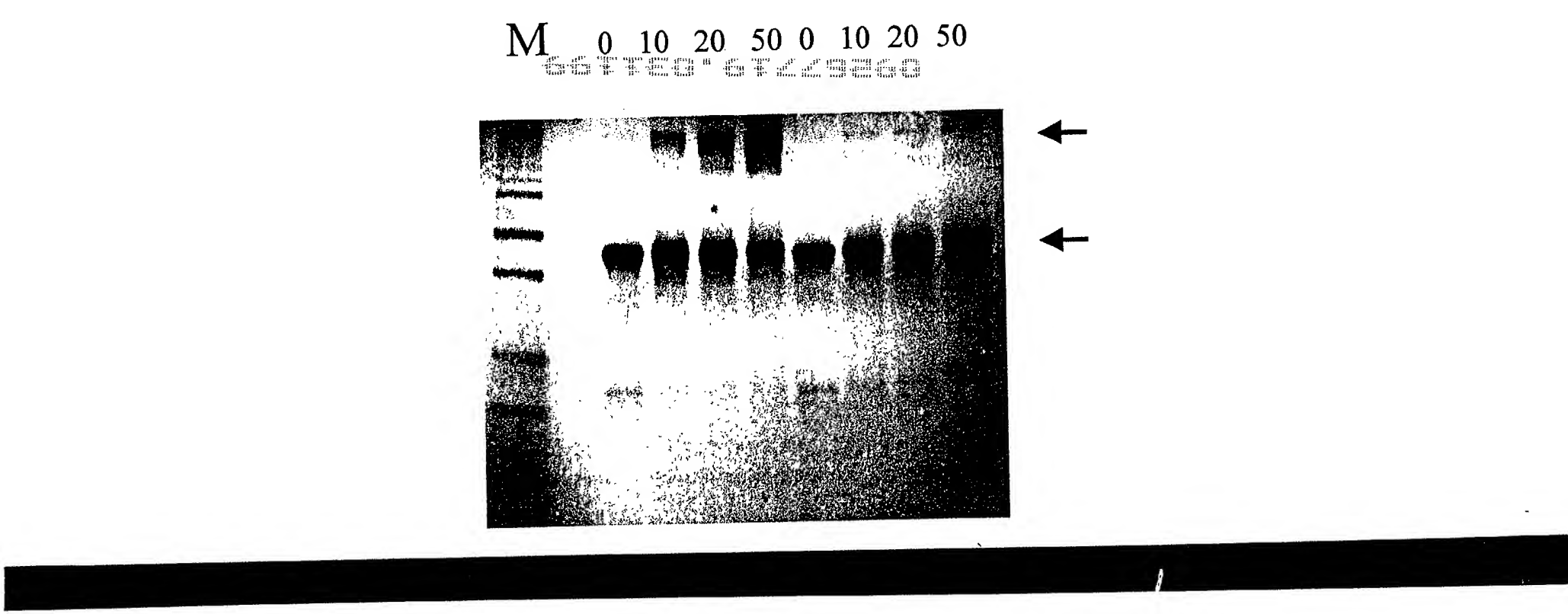


Figure 40 The Ara h 1 trimer is unstable at pH2.

In order to assess the stability of the Ara h 1 trimer at pHs that would be encountered in the human stomach, cross-linking experiments were performed using purified Ara h 1 protein suspended in a pH 2.1 buffer. Purified Ara h 1 (2 mM) was suspended in 500 µl of either a pH 2.1 buffer or a pH 7.6 buffer and allowed to incubate for one hour at room temperature. Cross-linking was performed using 5% DSP in DMF for varying lengths of time (10, 20, or 50 seconds). Results indicate that the Ara h 1 trimer is unstable at acidic pHs that would be encountered in the human stomach but that the monomer is stable at this pH. Further experiments indicate that the monomer is stable at pH 2.1 for greater than 8 hours at 37°C.

Figure 41 A
EXPRESSION OF RECOMBINANT Ara h 2 PROTEIN IN *E. Coli*

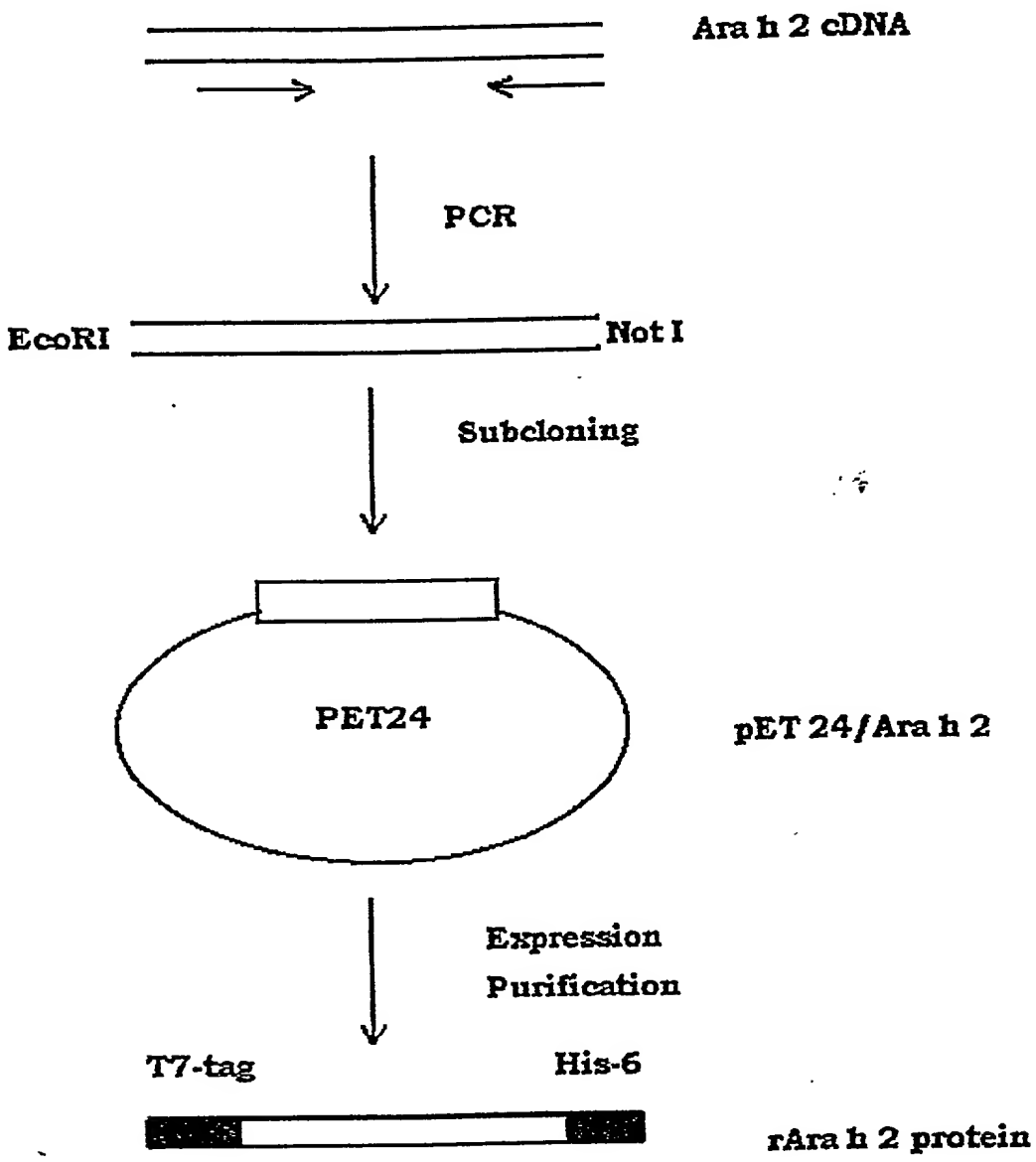
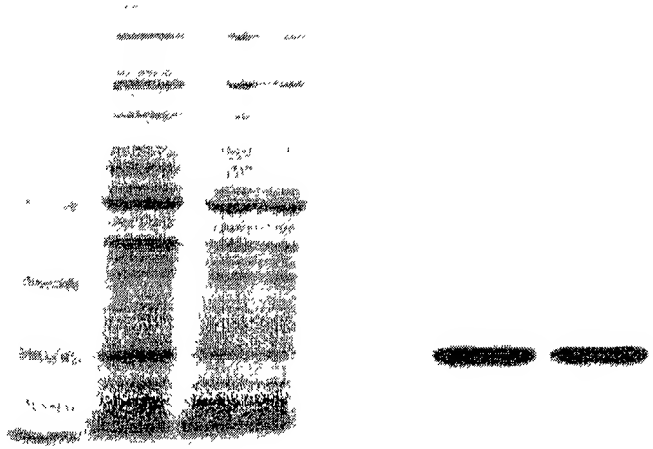


Figure 4/ B

PURIFICATION OF RECOMBINANT Ara h 2 PROTEINS ON A Ni-COLUMN



MW 1 2 3 4 5 6

Figure 42
INHIBITION OF IgE BINDING TO NATIVE Ara h 2 PROTEIN

0.5 ug of the native Ara h 2 protein purified from crude peanut extracts were loaded onto nitrocellulose membrane using a slot-blot apparatus. Membranes were incubated with patient serum pool (1:20) in the presence or absence of different concentrations of wild type or mutated recombinant Ara h 2 proteins. Membranes were probed for the bound IgE with ^{125}I anti-human IgE antibody. Laser densitometry of the autoradiograms was used to quantitate the relative amounts of IgE binding.

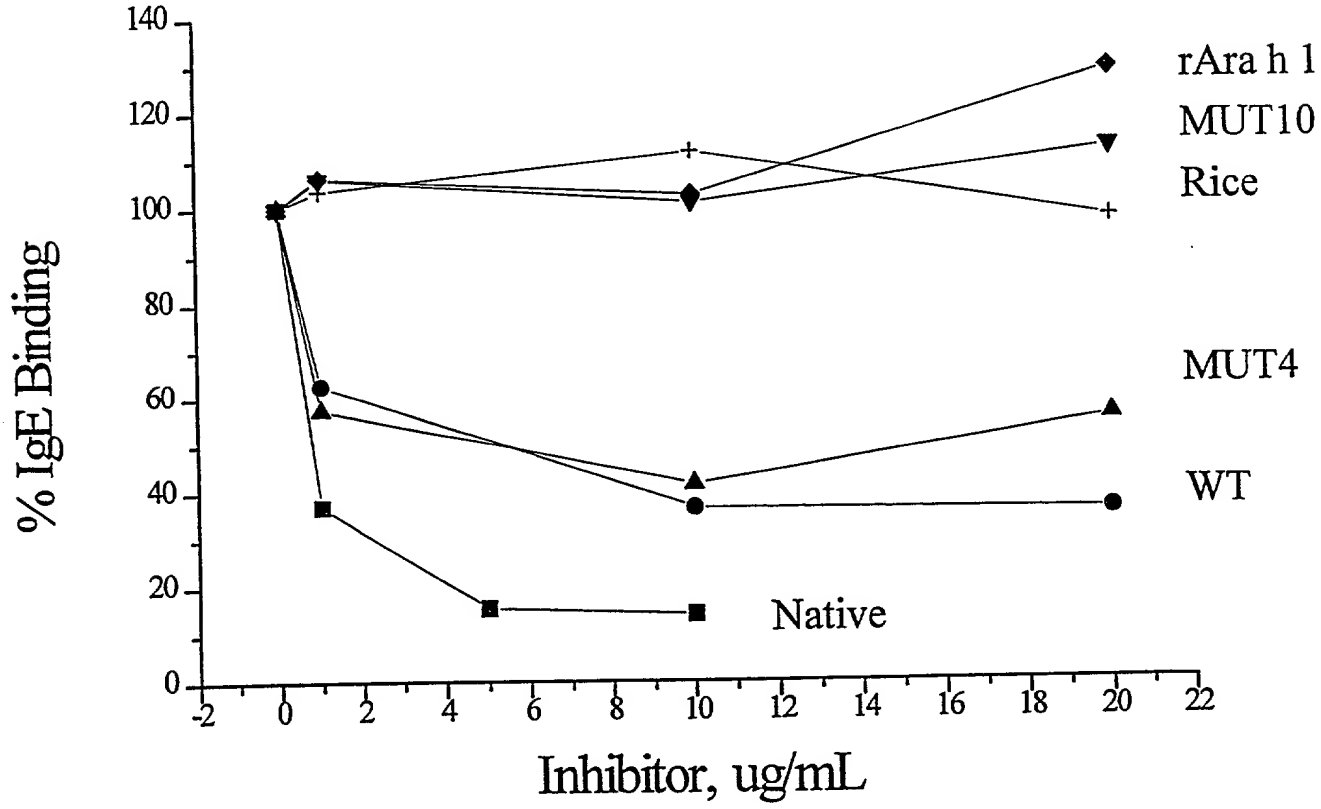
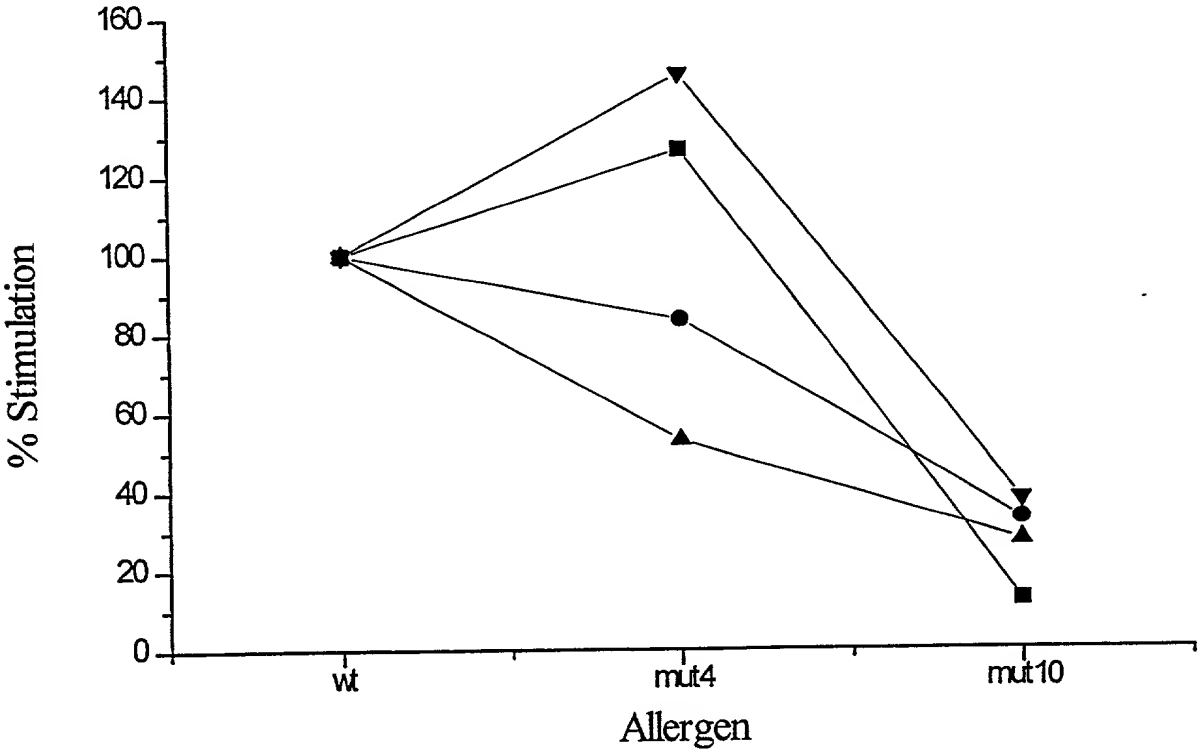
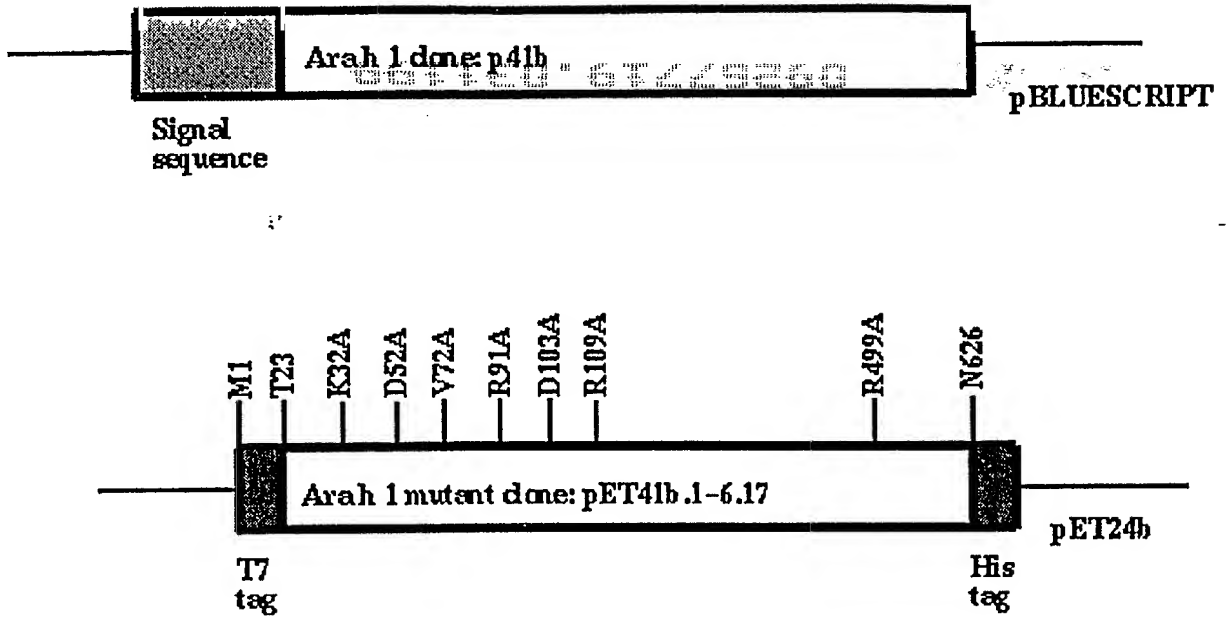


Figure 43
PROLIFERATION OF PBMCs FROM PEANUT SENSITIVE PATIENTS

PBMCs were isolated from heparinized venous blood of peanut-sensitive patients by density gradient centrifugation on Ficoll. $2x10^5$ cells per well were incubated in triplicates for 7 days in RPMI media with 5% human AB serum in the presence of 10 ug/ml of the native Ara h 2 protein purified from the crude peanut extract or recombinant Ara h 2 proteins purified from *E.coli.* Cells incubated in media only were used as a control. Proliferation was measured by the incorporation of tritiated thymidine. Stimulation index is calculated as a ratio of radioactivity for the cells growing in the presence of allergen to that for the cells growing in media alone.





Epitope	Mutation
1	K32A
2	D52A
3	V72A
4	R91A
5	D103A
6	R109A
17	R499A

Figure 11 Wild type and mutant Ara h 1 plasmid constructs.

The top diagram represents the original Ara h 1 p41b construct. It consists of the Ara h 1 cDNA insert withir PBLUESRIPT SK- vector. Noted are the Met1, Thr23, and Asn626 positions. The residues Met1-Thr23 repr the Ara h 1 signal sequence and Asn626 is the C-terminal residue. The lower diagram represents the mutantA 1 construct pET41b.1-6.17. It was constructed by inserting a PCR product into the pET24b expression v Noted are the epitope mutation positions (not drawn to scale) and the positions of the T7 and His tags. particular mutations within each epitope are listed under the diagrams.

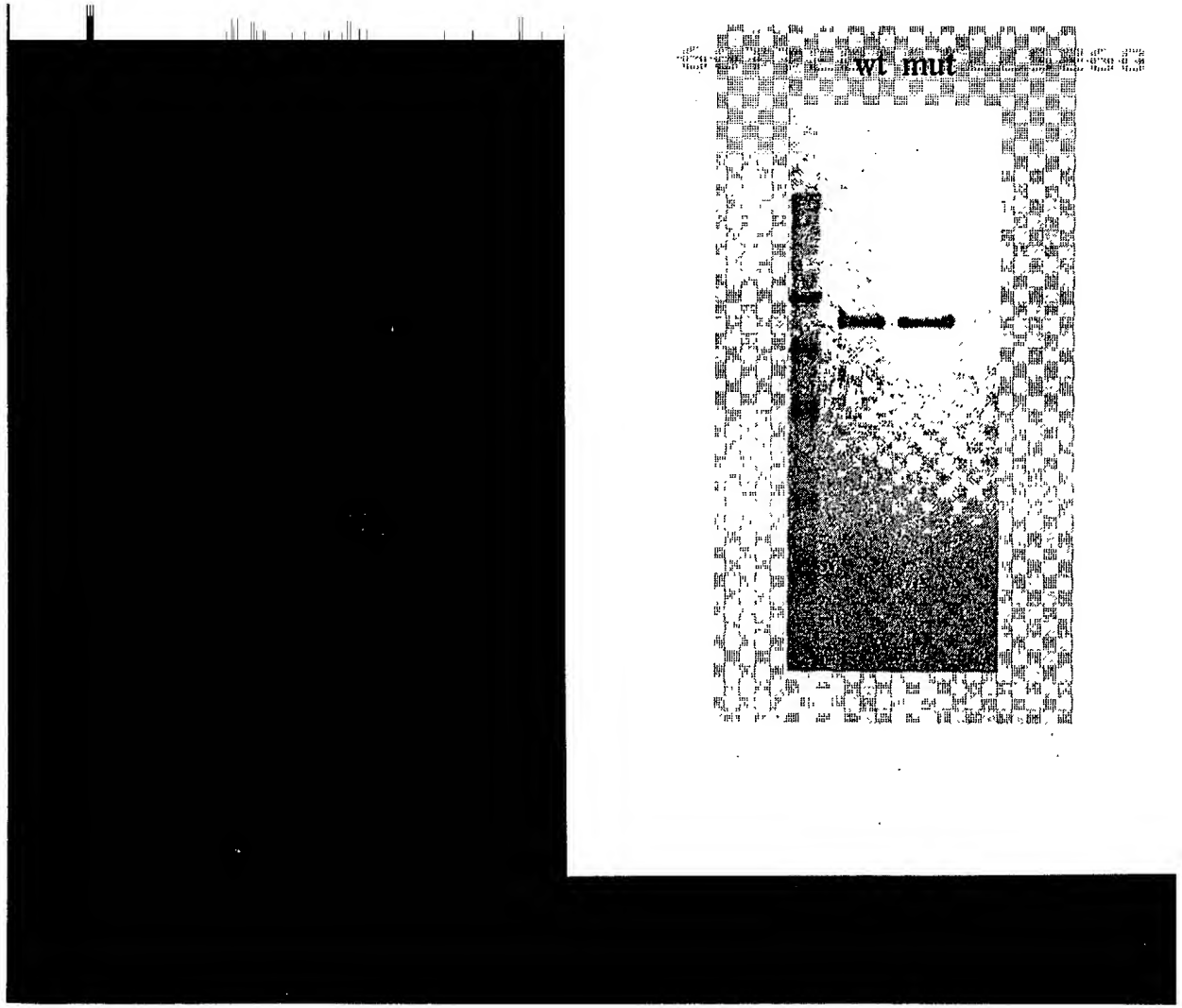
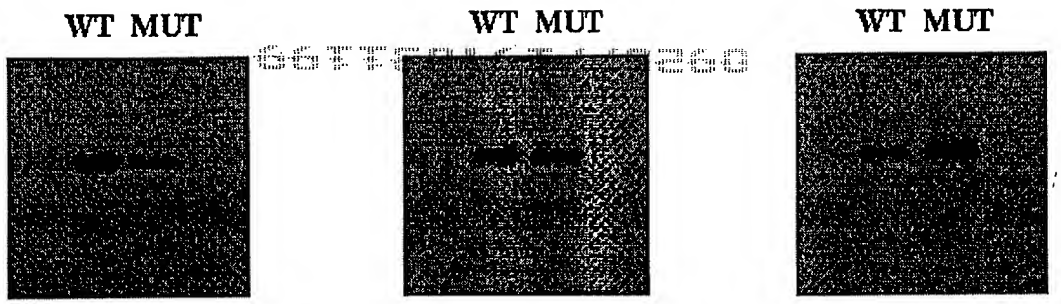


Figure 45 Wild type and mutant protein western blot control.

A western blot control was performed on the wild type and mutantAra h 1 recombinant proteins to ensure that an equal amount of each protein was used in these studies. Novex see-blue molecular weight markers were visualized by Coomassie staining in the first lane. Equal amounts of wild type (middle lane) and mutant (right lane) were detected by probing with anti-T7 antibody alkalinephosphatase conjugate. Both proteins migrate at their expected molecular weights (65 kDa).



Patient 1

Mutant epitopes:
1, 4, 5, 17

WT epitopes:
8, 13

Patient 2

Mutant epitopes:
2, 3, 4, 17

WT epitopes:
14, 18

Patient 3

Mutant epitopes:
4, 5, 17

WT epitopes:
11, 14, 18, 19, 20 ,22

Figure 46 Mutation of the Ara h 1 protein leads to altered IgE binding.

Three western blots of wild type (left lane) and mutant (right lane) recombinant proteins probed with indiv peanut-sensitive patient sera are shown. The epitopes that each patient recognized are indicated below each blot. Mutant epitopes corresponds to the epitopes that the patient recognized that were altered in the mutant protein. WT epitopes corresponds to epitopes that were recognized by the patient, but were not changed in the mutant protein. In the first panel IgE binding was decreased. In the second panel binding was roughly equal. In the third panel binding was increased.

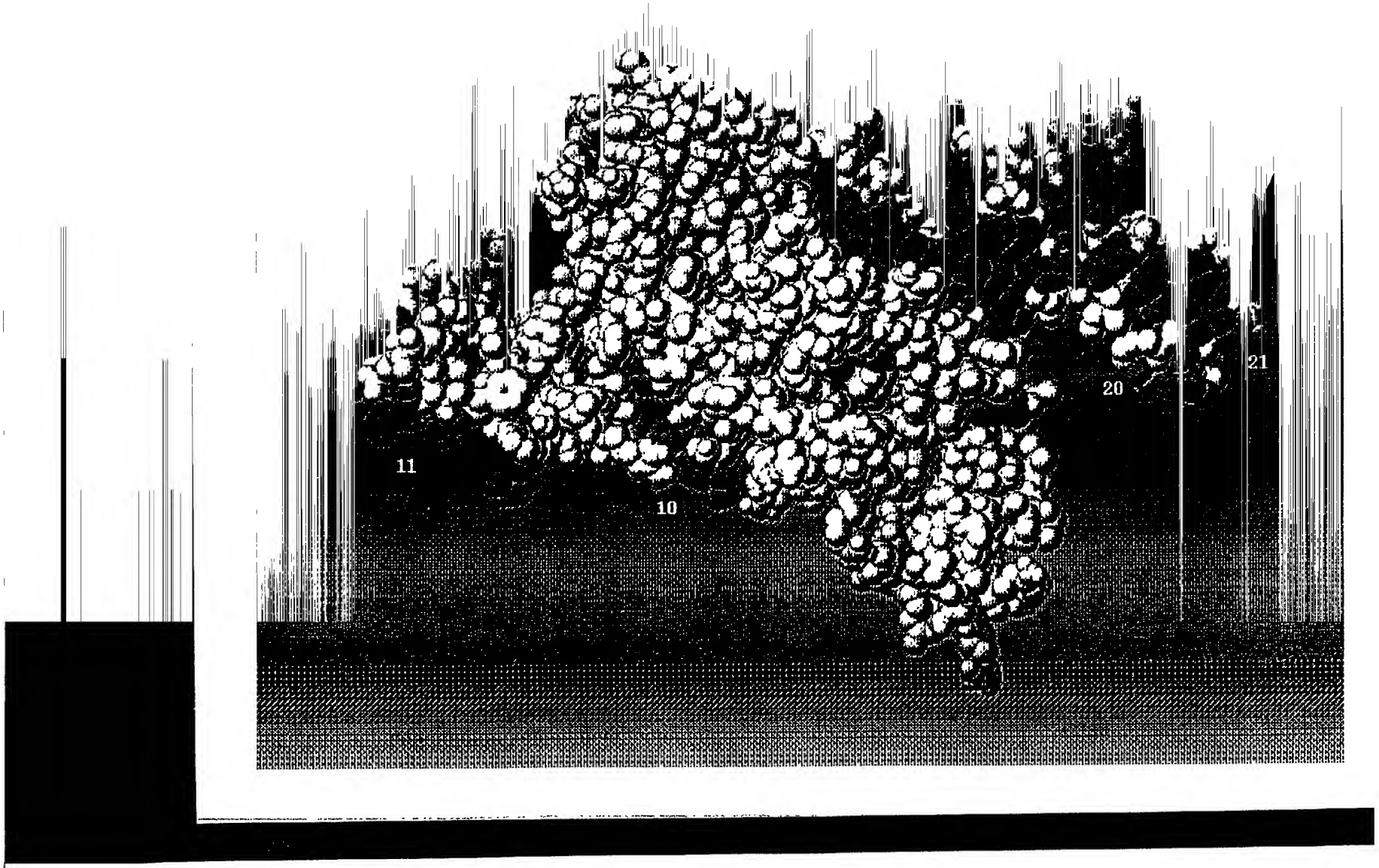


Figure 47 Tertiary structure model of the Ara h 1 protein.

A space-filled model of the middle and C-terminal domains of the Ara h 1 allergen is shown. The red ar represent the IgE binding epitopes. The yellow atoms represent residues that were determined to be critical for binding to occur. The numbers correspond to some of the epitopes listed in Table 1.

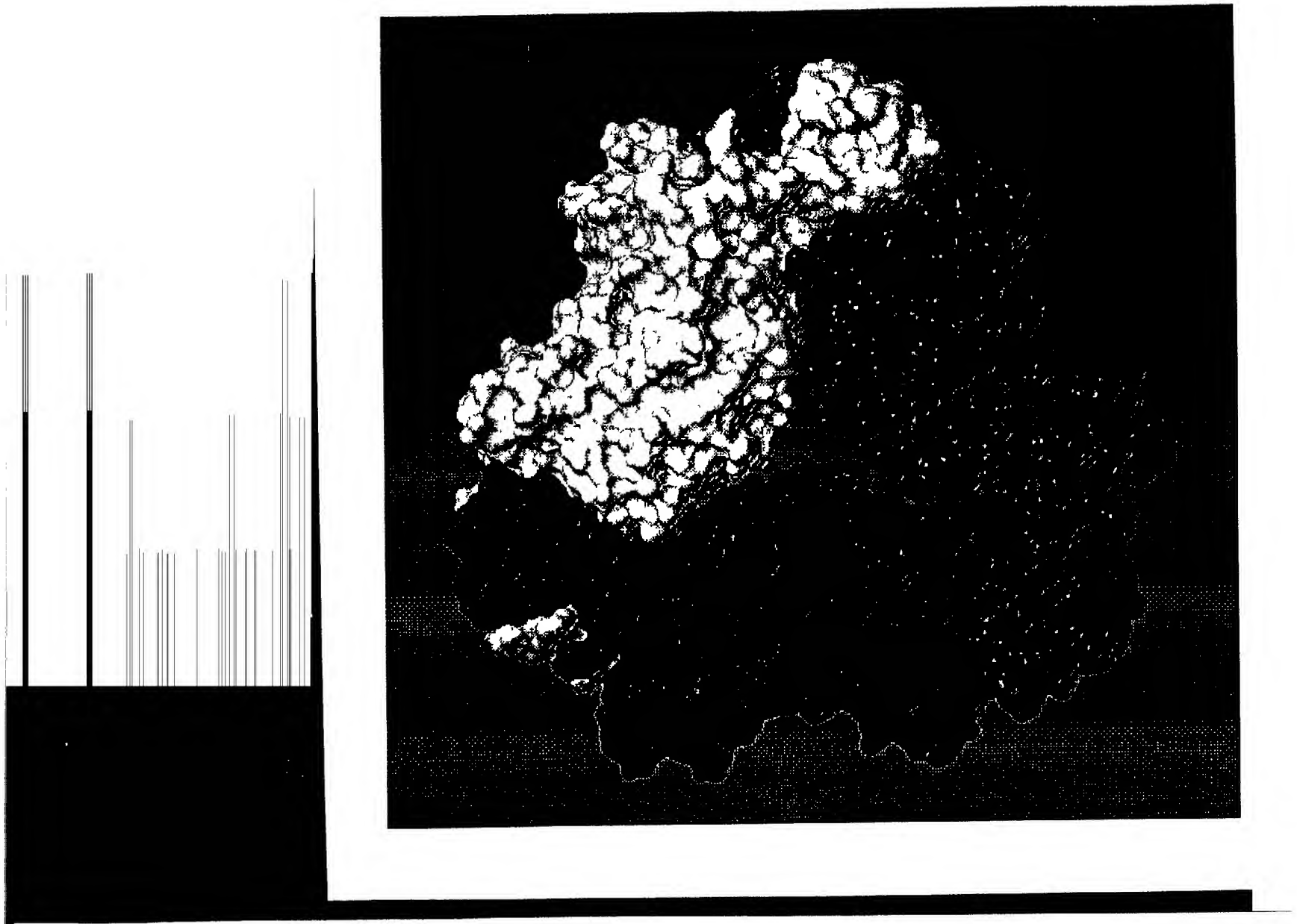


Figure 48 Contoured surface diagram of the trimeric Ara h 1 model.

A contoured surface of each Ara h 1 monomer is shown in a different color. The trimeric structure is based on the phaseolin trimer structure. The alpha helical bundles and adjacent beta sheets form the interface of monomer-monomer contact.

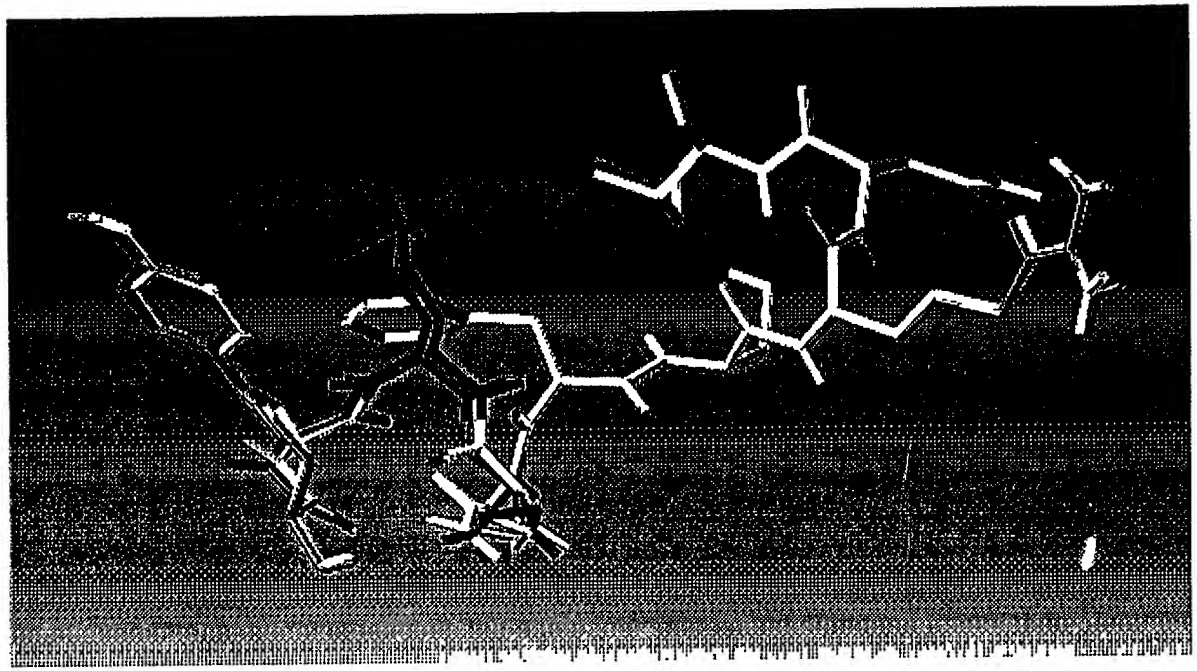


Figure 49 Calculated effect of L313M mutation on epitope 11.

A stick diagram of epitope 11 (SYLQGFSRNT) is shown in yellow with the exception ofLeu 313 which is shown in red. A mutant, L313M, Ara h 1 protein was computationally generated. The region corresponding to the mutant epitope 11 region is shown in white where the substituted residue (methionine) is shown in purple. The remaining atoms of both the wild type and mutant peptide are not shown for clarity. This mutation is not predicted to alter the structure of the mutant protein substantially.

FIGURE 50 Immunoblot of purified recombinant Ara h 3 with serum IgE from individual patients

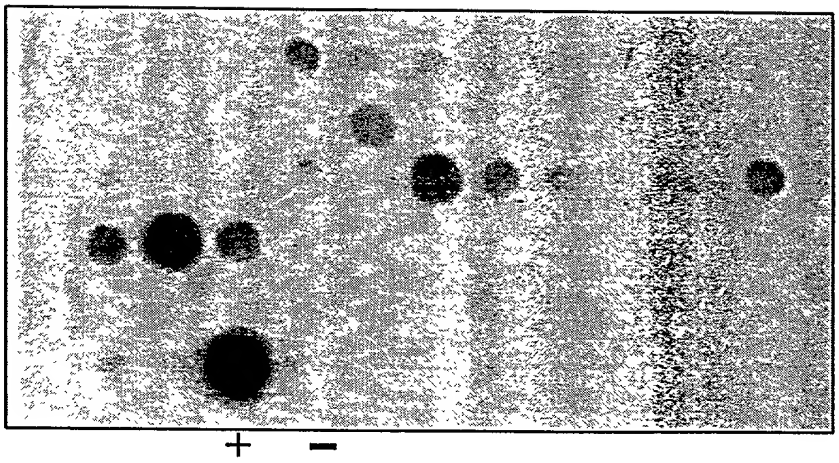
A B C D E F G H I J K L M N O P Q R S T

A

Ara h 3

Amino Acid

1-103
97-199
193-295
289-391
385-487
481-510



B

R1

ISFRQQPEEN ACQFQRLNAQ RPDNRIESEG GYIETWNPNN QEFECAGVAL 50

SRLVIRRNAL RRPFYSNAPQ EIFIQQGRGY FGLIFPGCPR HYEEPHTQGR 100

R2

RSQSQRPPRR LQGEDQSQQQ RDSHQKVHRF DEGDLIAVPT GVAFWLYNDH 150

DTDVVAVSLT DTNNNDNQLD QFPRRFNLAG NTEQEFLRYQ QQSRQSRRRS 200

R3

LPYSPYSPQS QPRQEEREFS PRGQHSRRER AGQEEENEGG NIFSGFTPEF 250

R4

LEQAFQVDDR QIVQNLRGET ESEEEGAIVT VRGGLRILSP DRKRRADEEE 300

EYDEDEYEYD EEDRRRGRGS RGRGNGIEET ICTASAKKNI GRNRSPDIYN 350

PQAGSLKTAN DLNLLILRWL GLSAEYGNLY RNALFVAHYN TNAHSIIYRL 400

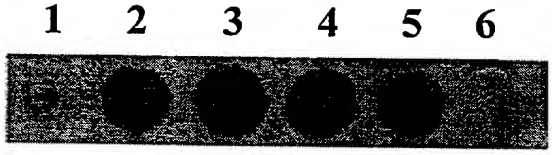
RGRAHVQVVD SNGNRVYDEE LQEGHVLVVP QNFAVAGKSQ SENFEYVAFK 450

TDSRPSIANL AGENSVIDNL PEEVVANSYG LQREQARQLK NNNPFKFFVP 500

PSQQSPRAVA 510

Fig. 51

A.


1 2 3 4 5 6

B.

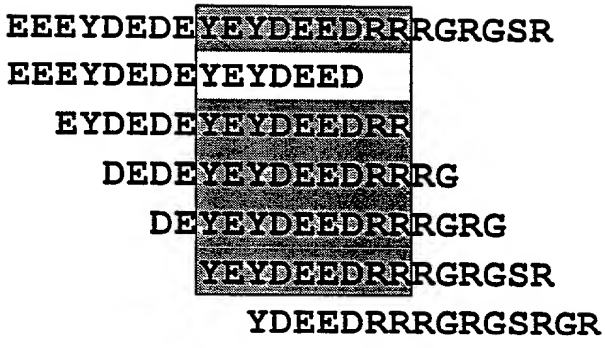

EEEYDEDEYEYDEEDRRRGRGSR
1. EEEYDEDEYEYDEED
2. EYDEDEYEYDEEDRR
3. DEDEYEYDEEDRRRG
4. DEYEYDEEDRRRGRG
5. YEYDEEDRRRGRGSR
6. YDEEDRRRGRGSRGR

Fig. 52

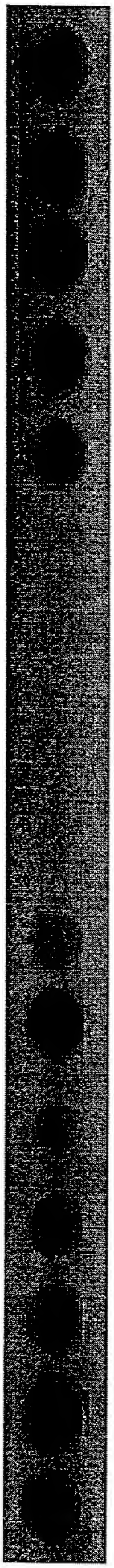

D303A
E304A
D305A
E306A
Y307A
E308A
Y309A
D310A
E311A
E312A
D313A
R314A
R315A
R316A
G317A
WT

Fig. 53

FIGURE 54 Recombinant expression and Western blot analysis of the Ara h 3 mutant

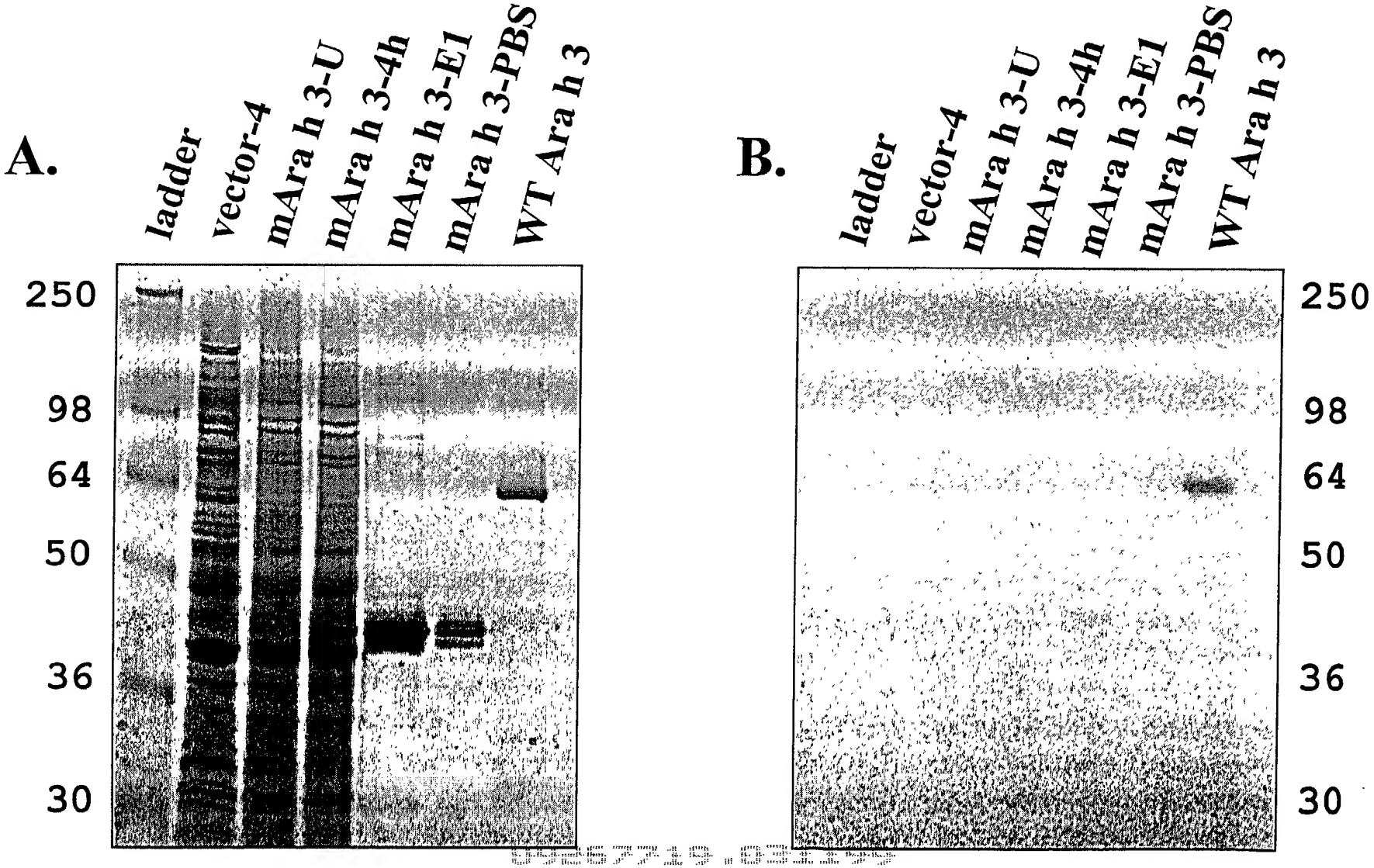


Fig. 55

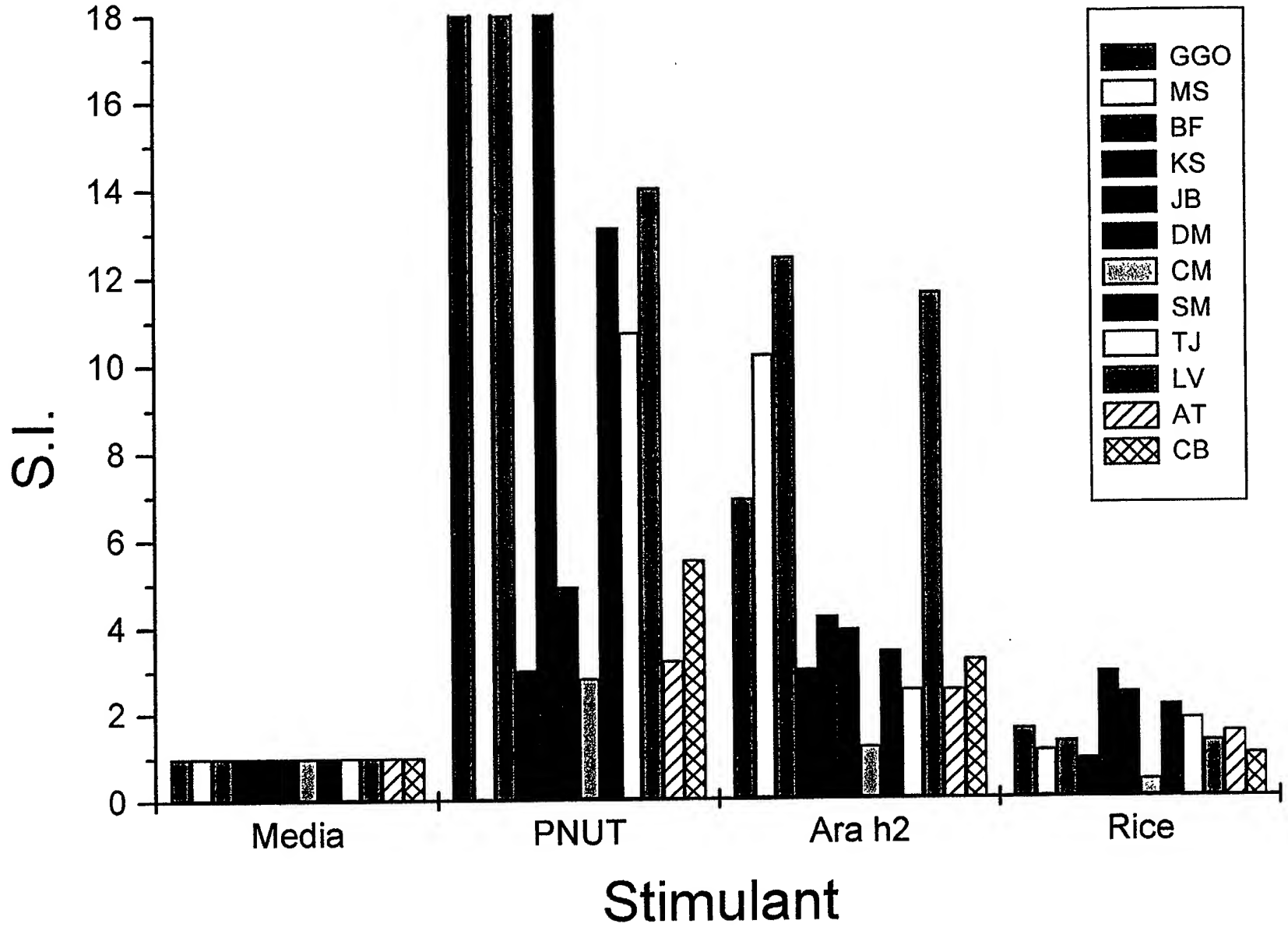
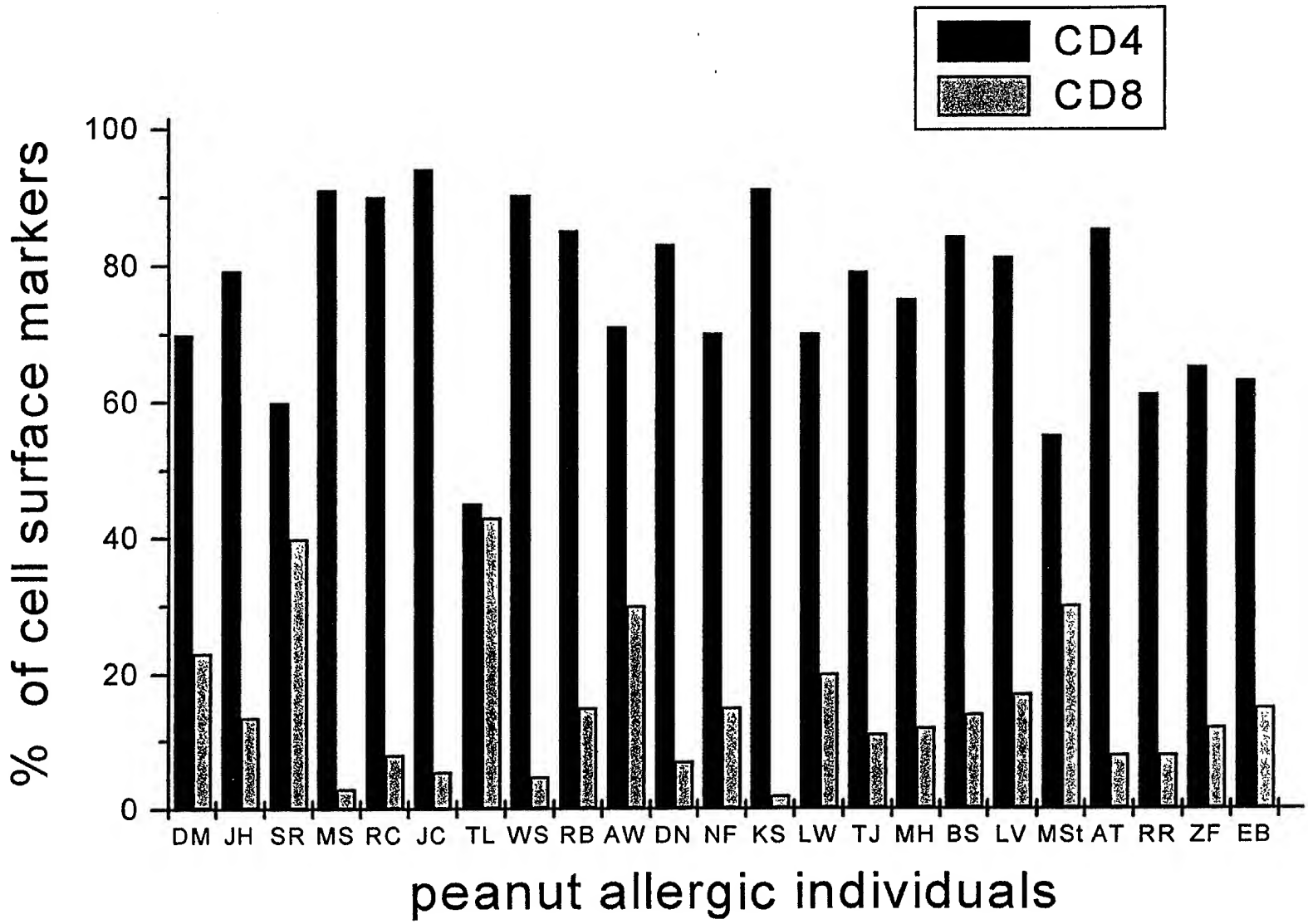

18
16
14
12
10
8
6
4
2
0
S.I.
Media
PNUT
Ara h2
Rice
Stimulant
GGO
MS
BF
KS
JB
DM
CM
SM
TJ
LV
AT
CB

Fig. 56


CD4
CD8
% of cell surface markers
100
80
60
40
20
0
DM JH SR MS RC JC TL WS RB AW DN NF KS LW TJ MH BS LV MSt AT RR ZF EB
peanut allergic individuals

Atty. Dkt. No.
ARK00898103A

DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

TERTIARY STRUCTURE OF PEANUT ALLERGEN ARA H 1

The specification of which

[X] is attached hereto.

[ ] Application Serial No. _____ filed _____.
amended on _____ (if applicable).

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, Section 1.56 (a).

I hereby claim foreign priority benefits under Title 35, United States Code, Section 119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application to which priority is claimed:

PRIOR FOREIGN APPLICATION(S)

			Priority Claimed	
			[ ]	[ ]
(Number)	(Country)	(Day/Month/Year filed)	Yes	No

I hereby claim the benefit under Title 35, United States Code, Section 120 of any United States application(s) or PCT international application(s) designating the United States of America that is/are listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in those/that prior applications in the manner provided by the first paragraph of Title 35, United States

EXPRESS MAIL-Mailing Label No. EL212162105US
Date of Deposit: 3-11-99
I hereby certify that this paper and fee is being deposited with the United States Postal Service "EXPRESS MAIL POST OFFICE TO ADDRESSEE" service under 37 C.F.R. 1.10 on the date indicated above and is addressed to the Assistant Commissioner for Patents; Washington, D.C. 20231

Code, Section 112, I acknowledge the duty to disclose information that is material to the examination of this application, namely, information where there is substantial likelihood that a reasonable Examiner would consider it important in deciding whether to allow the application to issue as a patent, which occurred between the filing date of the prior application(s) and the national or PCT international filing date of this application, as defined in Title 37, Code of Federal Regulations, Section 1.56 (a):

60/077,763	3/12/98	Pending
(Appl. Serial No.)	(Filing Date)	(Status - Patented, Pending, Abandoned)

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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